

AD_____

Award Number: W81XWH-04-1-0876

TITLE: Diversity, Replication, Pathogenicity and Cell Biology of Crimean Congo Hemorrhagic Fever Virus

PRINCIPAL INVESTIGATOR: Adolfo Garcia-Sastre, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, NY 10029

REPORT DATE: October 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20060516040

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE 01-10-2005		2. REPORT TYPE Annual		3. DATES COVERED 15 Sep 2004 – 15 Sep 2005	
4. TITLE AND SUBTITLE Diversity, Replication, Pathogenicity and Cell Biology of Crimean Congo Hemorrhagic Fever Virus				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0876	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Adolfo Garcia-Sastre, Ph.D.				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mount Sinai School of Medicine New York, NY 10029				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This research project is a result of a collaboration between three research groups aimed at elucidating basic replication processes of CCHFV with the expected outcome of providing basic research reagents and establishing the foundation of knowledge necessary for discovery of vaccines and antiviral therapeutics for Crimean Congo hemorrhagic fever. Our major findings during the first year of support are the following: We have sequenced and cloned in expression vectors the L, G and N viral proteins of CCHFV, strain 10200; we have cloned and expressed a mini-replicon RNA of CCHFV; we have expressed the N protein of CCHFV in bacteria and found RNA-binding activity; we have determined the processing and subcellular localization of the glycoproteins of CCHFV; we have sequenced the M gene of 5 divergent CCHFV isolates and found broadly reactive as well as group-specific neutralizing epitopes. Our results provide novel insights on the molecular biology of this understudied highly pathogenic human virus.					
15. SUBJECT TERMS CCHFV, bunyavirus, reverse genetics, virus receptor, animal model, arbovirus					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 35	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Cover.....	1
SF 298.....	2
Table of contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	13
Reportable Outcomes.....	13
Conclusions.....	14
References.....	14
Appendices.....	15

1. Introduction.

This investigator-initiated project represents a highly collaborative team approach to the study of CCHFV. This proposal is comprised of three subprojects. The body of the progress report for each sub-project is outlined in the following pages:

2. Body.

Subproject #1: Characterization of CCHFV strains and small animal model development

Work carried out under subproject #1 is being performed in the laboratory of Dr. Connie Schmaljohn at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID), and is directed at working with the intact virus in high biocontainment and supporting projects 2 and 3 with reagents and experiments with virus to complement in vitro work from subproject 2 and 3. The precise tasks are listed below:

Task 1. Determination of genetic factors of virulence by comparison of tick, livestock and human isolates of CCHFV (Years 1-3)

- a. Clone and sequence the complete genomes of 5 CCHFV isolates obtained from ticks, livestock or humans (Year 1)
- b. Perform viral growth curves, cytokine analysis, and preliminary gene expression profiling (Years 2-3)
- c. Refine gene expression analysis and integrate data from sequence determination, viral growth curves cytokine analysis, and gene expression analysis (Years 2-4)

Task 2. Epitope mapping of CCHFV monoclonal antibodies.

- a. Generate CCHFV specific peptide microarray (Year 1)
- b. Map epitopes of CCHFV specific monoclonal antibodies (Year 2)

Task 3. Development of a mouse-model for CCHFV.

- a. Test CCHFV strains for growth in suckling mice (Years 1)
- b. Adapt CCHFV strains for growth in adult mice (1-4)

Task 4. Support using live virus for projects 2 and 3 at USAMRIID

- a. Support, confirm, and expand scientific developments from project 2 and 3 with live virus work (Years 1-4)

The progress is noted below the specific task.

Task 1. Determination of genetic factors of virulence by comparison of tick, livestock and human isolates of CCHFV (Years 1-3)

- a. Clone and sequence the complete genomes of 5 CCHFV isolates obtained from ticks, livestock or humans (Year 1)**

Many of the prototypical laboratory strains of CCHFV were recently sequenced by a group from CDC. We are in the process of obtaining novel isolates from countries in the former Soviet Union. This will prevent unnecessary duplication.

- b. Perform viral growth curves, cytokine analysis, and preliminary gene expression profiling (Years 2-3)**

A tick isolate (IBr10-200) and human isolate (3010) have been compared in a macrophage cell line. The tick isolate is restricted for growth while the human isolate was able to productively infect the cells. Viral culture medias are various time post infection were harvested and cell free supernatants were prepared. These samples were assayed for viral titers by real time polymerase chain reaction and by plaque assay. Additionally, samples were tested using the BIORAD multiplex cytokine bead array system. Cellular cytokine levels for 16 different cytokines were determined. The current system is not efficient for viral replication. We are in the process of identifying a more efficient substrate for viral replication and will repeat the experiment. There are a number of cell substrates available.

c. Refine gene expression analysis and integrate data from sequence determination, viral growth curves cytokine analysis, and gene expression analysis (Years 2-4)

This experiment will be performed when an efficient cell substrate has been identified.

Task 2. Epitope mapping of CCHFV monoclonal antibodies.

a. Generate CCHFV specific peptide microarray (Year 1)

We are using an overlapping peptide library for N and G. This peptide technology allows for both highly paralleled detection of epitopes and throughput. Peptides have been ordered.

b. Map epitopes of CCHFV specific monoclonal antibodies (Year 2)

No work done.

Task 3. Development of a mouse-model for CCHFV.

a. Test CCHFV strains for growth in suckling mice (Years 1)

We have tested 6 strains of CCHFV in suckling mice by I.P. injection. The IBR 10-200 strain is the most virulent in suckling mice. We will attempt to adapt this strain to adult mice. This finding is not surprising. IBR 10-200 has been passaged 12 times in suckling mouse brain prior. Other strains of CCHFV tested in this experiment have only been passaged no more than 4 times in suckling mouse brain.

b. Adapt CCHFV strains for growth in adult mice (1-4)

No work done.

Task 4. Support using live virus for projects 2 and 3 at USAMRIID

a. Support, confirm, and expand scientific developments from project 2 and 3 with live virus work (Years 1-4)

1. Louis Altamura from Dr Doms's laboratory has successfully completed: security risk assessments, enrolled in the special immunization program, and completed laboratory training at USAMRIID. He is able to function as an independent investigator without direct supervision.

2. We have supplied Dr Doms's laboratory with purified and irradiated viral antigen, monoclonal antibodies, and genomic material.

3. We have supplied Dr Garcia-Sastre's laboratory with genomic material

4. We have performed intact virus experiments in support of the reverse genetic system. Specially we have optimized transfection methods for a cell substrate that is highly susceptible to CCHFV infection. Also we have demonstrated that the viral minigenome construct can recapitulated genomic transcription and replication by transcomplementation using a viral polymerase supplied by a co-co-infecting virus.

Subproject #2: Reverse genetics of CCHFV

Subproject #2 focuses on understanding the molecular interactions between the components of the RNA replication machinery of CCHFV: the N and L proteins and the viral RNAs, with the final goal of establishing reverse genetics techniques for the rescue of CCHFV from plasmid DNA. These techniques will be used to generate attenuated strains of CCHFV. There are three tasks:

Task 1. Characterization of cis- and trans-acting signals involved in RNA replication and transcription of CCHFV (Years 1-3).

Task 2. Generation of recombinant CCHFV from cDNA (Year 3).

Task 3. Generation of attenuated CCHFV by reverse genetics (Year 3).

Over the first year, we have made the most progress with *Task 1*.

Sequencing and cloning of the large (L) gene

We completed the genome sequencing of the large RNA segment of CCHFV, strain 10200. The gene contains 12,160 nucleotides, with a 76 nucleotide long 3' UTR and a 246 long 5' UTR. A single ORF was identified, encoding for a 3,945 amino acid protein. After obtaining the sequence, two other groups also reported sequences from the L gene of CCHFV (3, 4). Our sequence is identical to that of the other two groups except for a few amino acid changes. The significance of these changes will be studied in the near future. The protein sequence showed the core polymerase motifs characteristic of the RNA-dependent RNA polymerases of segmented negative-stranded viruses and in the amino terminal portion, an ovarian tumor (OTU)-like protease motif was identified (Fig.1)

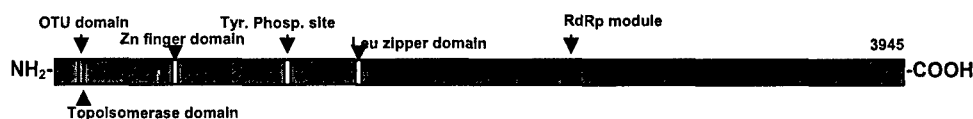


Figure1: Predicted domains present in CCHFV L protein

The L cDNA was cloned from mRNA extracted from supernatant of CCHFV-infected cells. The chosen vector for assembly was pGEMT and from it, the L was subcloned into other expression vectors. Due to the lack of an optimal antibody against the full length L protein, we have constructed different tagged version of the plasmids (Table 1).

Plasmid	Tag	Characteristic of the vector
pGEMT-L	None	T7 promoter and SP6 promoter
pTM1-L	None	T7 promoter and IRES
pCAGGS-L-HA	Ha	Strong chicken β -actin promoter
pCAGGS-HA-L-HA	Flag	Strong chicken β -actin promoter
pCAGGS-L-Flag	Flag	Strong chicken β -actin promoter
pCAGGS-HA-L-Flag	Ha and Flag	Strong chicken β -actin promoter

Table1: List of plasmids containing the full length L cDNA

Expression of the L protein

The L protein has been successfully expressed in several mammalian cell lines. Western blot and immunofluorescence assays followed by confocal microscopy, showed a 450kDa protein with cytoplasmic localization, as expected by primary sequence analysis (Fig 2).

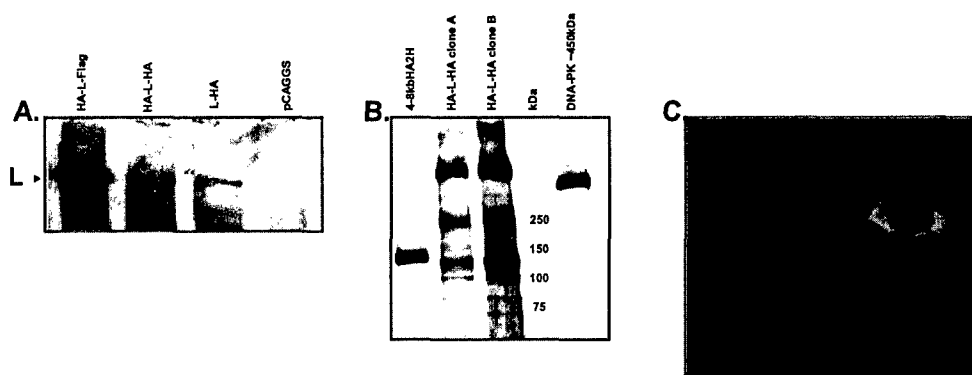


Figure 2: **Expression of full length L protein.** Left: Western blot against HA from total cell lysate (293T cells). Center: Immunoprecipitation and western blot using α -HA antibody. Right: Immunofluorescence and confocal microscopy in HeLa cells (using α -Ha (green) and DAPI staining)

Minireplicon system for CCHFV

Polymerase I system: We have constructed a reporter plasmid containing the firefly luciferase gene (in negative sense) flanked by the UTR of the S segment under the control of the human PolI promoter. The precise end of the transcript is achieved by adding the sequence of the hepatitis delta ribozyme. The functionality of this construct has been successfully tested by our collaborators at USMARIID in a CCHFV-driven minireplicon assays (Table 2). The results are consistent with those of Flick et al (2).



Figure 3: Schematic of the CCHFV pPOL-S-Luc reporter plasmid

Dilution	PolI	PolI + CCHFV	PolI-S-Luc	PolI-S-Luc + CCHFV
Neat	748	294	1996	9024
-1	552	300	1274	29048
-2	492	298	918	3104
-3	418	298	748	1802

Table 2: Minireplicon assay in 293T cells, 72 hours post infection with CCHFV

T7 RNA polymerase system: We have generated reporter plasmids containing the GFP or the CAT gene (in negative sense) flanked by the UTRs of the S and L segments under the control of a truncated T7 promoter and the corresponding terminator sequence. The precise end of the transcript is achieved by adding the hepatitis delta ribozyme coding sequence.

Constructed plasmids containing the small (S) segment cDNA

The S segment was cloned into a pGEMT vector and then subcloned into different expression plasmids (Table 3)

Plasmid	Tag	Characteristic of the vector
pGEMT-N	None	T7 and SP6 promoter
pTM1-N	None	T7 promoter and IRES
pCAGGS-N	None	Strong chicken β -actin promoter
pCAGGS-N-HA	HA in C-terminal	Strong chicken β -actin promoter
pCAGGS-Flag-N	Flag in N-terminal	Strong chicken β -actin promoter
pCAGGS-N-Flag	Flag in in C-terminal	Strong chicken β -actin promoter
pGEX-2T-N	GST in N-terminal	For bacterial expression
pGEX-6P1-N	GST in N-terminal	For bacterial expression

Table 3: List of plasmids containing the full length N cDNA

Expression of the nucleocapsid (N) protein

The N protein has been successfully expressed from all the plasmids described above. When analyzed by western blot, the N protein migrates at the molecular sized of ~50kDa. Over expression of the N protein in Hela cells and confocal microscopy showed a filament-like structure in the cytoplasm of the cells (Fig.4 left). The N protein, fused to GST, has been expressed and purified from the BL21 strain of E.Coli and a good yield was obtained. (Fig. 4, right).

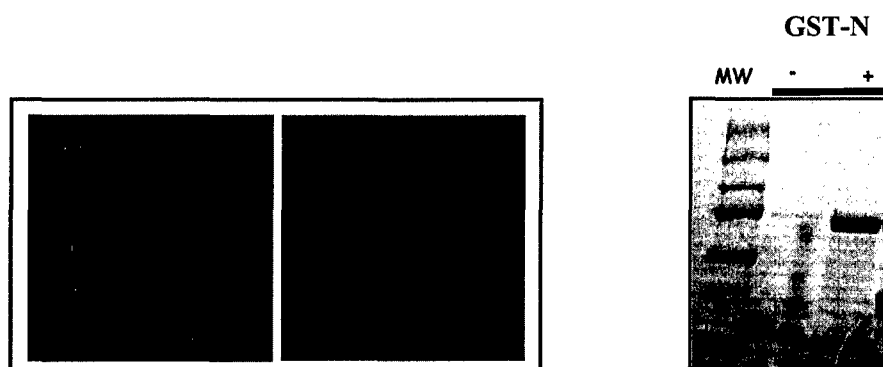


Figure 4: **Expression of the N protein.** Left: Confocal microscopy in Hela cells using monoclonal antibody (red) against N (α N 9D5). DAPI nuclear staining is shown in blue. Right: Coomassie staining of purified GST-N from bacterial extracts. The 75kDa migration corresponds to the size of the fused protein.

RNA binding ability by the N protein

We have demonstrated the ability of the N protein of CCHFV to interact and bind single stranded RNA. A dot blot assay has been performed using purified GST-N protein and P32 labeled ssRNA to show ssRNA binding (Fig. 5). Gel electrophoretic mobility shift assays (GEMSA) gave similar results (data not shown)

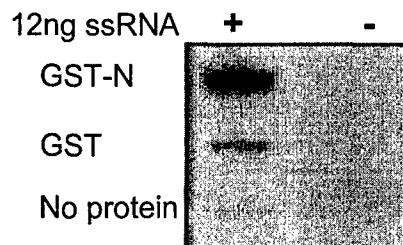


Figure 5: RNA binding by GST-N of CCHFV. Dot blot showing RNA binding by GST-N and not by GST.

Subproject #3: Cell Biology of CCHFV Glycoproteins

Work carried out under subproject #2 is being performed in the laboratory of Dr. Robert W. Doms at the University of Pennsylvania, and is directed towards characterizing the envelope glycoproteins of CCHFV. There are two tasks:

Task 1. Analyze the assembly and processing of CCHFV G1 and G2 glycoproteins and characterize the mucin-like domain present at the amino terminus of the polyprotein. (Years 1-3)

Task 2. Identify cell receptors and attachment factors for CCHFV.

Over the first year of this DOD-sponsored project, one paper has been published in Journal of Virology, and a second paper is in press at Journal of General Virology. Both of these studies were done in collaboration with our colleagues at USAMRIID and Mt. Sinai, and copies of each paper are included as Appendix material. Thus far, we have made the most progress with *Task 1*, though we have developed an assay that should enable us to make progress on *Task 2* as well.

***Task 1.* Analyze the assembly and processing of CCHFV G1 and G2 glycoproteins and characterize the mucin-like domain present at the amino terminus of the polyprotein. (Years 1-3)**

Characterize the localization of G1 and G2 when expressed in cells alone and in combination. This section of the grant has been completed and published (1). Briefly, to understand the processing and intracellular localization of the CCHFV glycoproteins as well as their neutralization and protection determinants, we produced and characterized monoclonal antibodies (MAbs) specific to both the G_N and G_C proteins. Using these MAbs, we found that G_N predominantly colocalized with a Golgi marker when expressed alone or with G_C, while G_C was transported to the Golgi only in the presence of G_N. Both proteins remained Endo H sensitive, indicating that the CCHFV glycoproteins are most likely targeted to the *cis* Golgi. Golgi targeting information partly resides within the G_N ectodomain, because a soluble version of G_N lacking its transmembrane and cytoplasmic domains also localized to the Golgi.

Perform mutational analysis of G1 and G2 to identify signal that are responsible for protein targeting. In the relatively few *Bunyaviridae* glycoproteins that have been examined, the Golgi localization signal has been localized to G_N, generally in the cytoplasmic tail (CT) or transmembrane domain (TM) of the protein. The C-terminal domain of G_N contains a stretch of predicted transmembrane domains and cytoplasmic loops between the first amino acid of G_C and the predicted C-terminus of G_N (7). The function of this unusual region on glycoprotein processing or any other step of the viral replication and cell cycle is unknown. To analyze the role of this region on G_N localization, we deleted two (G_N 2TM) or three (G_N 1TM) of the predicted four transmembrane domains at the G_N C-terminus. The resulting constructs, when transiently expressed in HEK293T cells and analyzed by Western blot analysis using the V5 tag

located at the N-terminus of the protein, showed the molecular weights expected for that of the G_N precursor. In addition, HeLa cells were transfected with the constructs and analyzed by IF. We found that all of the C-terminally truncated G_N constructs localized to the Golgi. These results suggest that the Golgi localization signal is not located in this region.

We next designed constructs that lacked the transmembrane domain of G_N and the cytoplasmic tail (sG_N) or that lack only the cytoplasmic tail (G_N -noCT). Both sG_N and G_N -noCT, although also present in the ER, localized to the Golgi when analyzed by immunofluorescence microscopy using MAbs against G_N . In addition, small amounts of sG_N were secreted in the media of cells transfected with this construct. Neither the full-length G_N and G_C nor the sG_C lacking its respective TM and CT domains were secreted in the medium. Interestingly, when a MAb against G_N (8F10) was used to immunoprecipitate sG_N in the presence of sG_C from cell lysates, sG_C was coimmunoprecipitated. Moreover, sG_N was retained inside the cell if co-expressed with sG_C , but was still secreted if co-expressed with an irrelevant protein. These results indicate that the interaction of G_C and G_N occurs through their ectodomains, that the proteins can fold correctly when in their soluble forms, and that Golgi targeting information resides within the ectodomain of G_N .

Development of polyclonal antibodies specific to CCHFV glycoproteins. Although an extensive panel of CCHFV specific monoclonal antibodies (mAbs) was developed at USAMRIID, very few of these mAbs are useful for western blotting (1). Furthermore, the precise epitopes of these antibodies have not been mapped, thereby making interpretations of glycoprotein processing difficult. To overcome these limitations, we sought to produce polyclonal antisera directed against the various domains of the CCHFV IbAr10200 M segment encoded polyprotein. In collaboration with ProSci, Inc., we synthesized 20 amino acid peptides derived from the primary sequence of the M polyprotein. These peptides represented the following domains: mucin, P35, G_N ectodomain, cytoplasmic domain #1, the lumenal domain, and cytoplasmic domain #2. Two rabbits were immunized with each of these peptides and their antisera were pooled and affinity purified with the immunizing peptides. Although all of the antisera was reactive towards the immunizing peptides by ELISA, only the G_N ectodomain antisera was useful in western blotting assays.

We also developed a series of thioredoxin fusion proteins for use as immunogens. DNA sequences encoding 100 amino acid polypeptides derived from the M polyprotein were cloned into the pBAD/Thio-TOPO vector system (Invitrogen) and expressed in bacteria to yield N-terminal thioredoxin fusion proteins with these domains. This panel of proteins represented the P35, G_N ectodomain, cytoplasmic domain #1, cytoplasmic domain #2, and G_C ectodomains. One rabbit was immunized with each of these proteins. We only obtained antisera useful for western blotting specific to the two cytoplasmic domains from this group.

Characterization of CCHFV glycoprotein oligomerization. The surface glycoproteins, G_N and G_C , are responsible for CCHFV attachment to and entry into cells. However, the mechanisms underlying these events and their structural organization on the virion remain undefined. To this end, we have investigated oligomerization of G_C in transfected cell lysates. We found that G_C migrated as both monomeric and oligomeric forms in nonreducing SDS-polyacrylamide gels, with the oligomeric form being preferentially recognized by a conformation-dependent monoclonal antibody. These oligomers are exceedingly stable as they are resistant to SDS, heat, reducing agents, and urea. We also employed crosslinking assays, equilibrium sedimentation, and co-immunoprecipitation studies to confirm G_C oligomerization. The G_C ectodomain is sufficient for oligomer formation, because G_C lacking its transmembrane domain and cytoplasmic tail also formed oligomers. This experiment argues against the possibility that G_C oligomers are an artifact of proteins localized to the same lipid membrane microdomain. G_C oligomers were formed both in the presence and absence of G_N expressed *in cis* or *in trans*, although oligomers migrate as slightly larger species in the presence of G_N . G_N coexpression is required for G_C transport to the Golgi, and so these differences in molecular weight may be attributable to complex glycosylation occurring only in the Golgi. Current efforts are focused on confirming the putative roles of trafficking and glycosylation upon the differences among these oligomers.

Preliminary data for this project relate to G_N oligomerization and the presence of glycoprotein oligomers in virions. Expression of a soluble version of the G_N ectodomain in transfected cells led to the formation of oligomers as evidence by their migration in non-reducing SDS-polyacrylamide gels. Further efforts are underway to confirm the formation of G_N oligomers and to investigate their ability to incorporate into hetero-oligomers with G_C . Confirmation of glycoprotein oligomerization in virions has been possible through Louis Altamura's access to BSL-3 facilities at USAMRIID. Louis was able to inactivate CCHFV virus stocks by gamma-irradiation for use at BSL-2 conditions. We have been able to demonstrate that G_C oligomers exist in CCHFV and are currently in the process of characterizing these oligomers in comparison to those found in transfected cell lysates.

Identification of novel C-terminal cleavage of G_N . Cotranslational proteolytic cleavage of the M genome segment encoded polyprotein yields two intermediate precursors, Pre- G_N (140 kDa) and Pre- G_C (85 kDa), that are further processed by cellular proteases to generate the mature G_N (38 kDa) and G_C (75 kDa). Although the precise N-termini of the G_N and G_C ectodomains have been defined, a number of putative cleavage sites within Pre- G_N remain to be confirmed and characterized. We have focused recent efforts on characterizing C-terminal cleavage of Pre- G_N in order to define the mature G_N C-terminus. Previous work in our lab has shown that a C-terminally epitope tagged Pre- G_N construct ending at amino acid 961 underwent C-terminal cleavage to generate an approximately 20-25 kDa fragment. In order to identify the site of this cleavage event, we employed deletion and truncation mutagenesis strategies in hopes of removing the cleavage motif and thereby preventing generation of the C-terminal fragment. Deletions and truncations were performed in the cytoplasmic and luminal domains immediately following the G_N ectodomain. Deletions within the first cytoplasmic domain did not affect C-terminal cleavage of Pre- G_N but did decrease the size of mature G_N , suggesting that this loop is contained within the mature glycoprotein. Deletions with the subsequent luminal domain had no impact on C-terminal cleavage or the size of mature G_N , indicating that this domain is not found within the mature glycoprotein. Truncation mutants immediately preceding (amino acid 841) or immediately following (amino acid 856) the luminal domain did not generate a C-terminal fragment and prevented Pre- G_N processing to yield mature G_N . Further studies are underway to characterize the role of the transmembrane domains in the generation of the C-terminal fragment and the potential for cleavage motifs to be contained within them.

Genotypic and antigenic characterization of CCHFV glycoproteins. Despite the fact that the geographic distribution of CCHFV is quite broad, limited sequence information is available on the viral M segment, with sequences of isolates obtained outside of China or Russia being rare. Nonetheless, available information indicates that both G_N and G_C can exhibit significant sequence diversity. This, coupled with the fact that only the Martin strain of CCHFV has been used in studies on the processing of CCHFV G_N and G_C (5, 7), provides a strong rationale for cloning and sequencing diverse CCHFV isolates, and comparing the cell biology of divergent G_N and G_C proteins. Our colleagues at USAMRIID have assembled a panel of CCHFV isolates from ticks, livestock infected by ticks, humans infected by ticks, humans who contracted the disease by contact with infected livestock, or humans who contracted the disease from another human. Strains include two isolates from human cases of CCHFV in South Africa (SPU 41/84 and SPU 94/87), two strains from Uzbekistan (U2-2-002 and M-20, a strain that was isolated from a fatal human case), and a strain isolated from *Hyalomma asiaticum* tick in China (Hy13). Viral RNA was extracted from cell lysates of infected cells after which first-strand cDNA was generated using Superscript II reverse transcriptase (Invitrogen, CA). A series of primers generated from consensus alignments of known CCHFV M segments were used in PCR to generate the 5' 3.5 Kb and 3' 3 Kb of the M segment using Taq High-Fidelity polymerase. These fragments share 1Kb of overlapping sequence. Fragments were TA subcloned (pCR4, Invitrogen, CA) and sequenced. Using this strategy, the M segment full-length sequence of SPU 41/84, Uzbek U2-2-002, and Hy13 strains were obtained. We fully sequenced the M segments and expressed the glycoproteins derived from the new viral isolates. These new sequences showed high variability in the N-terminal region of G_N and more modest differences in the remainder of G_N and in G_C . Phylogenetic analyses placed these newly identified strains in three of the four previously described M segment groups. Studies with a panel of monoclonal antibodies specific to G_N and G_C indicated that there were significant antigenic differences

between the M segment groups, though several neutralizing epitopes in both G_N and in G_C were conserved among all strains examined. Thus, the genetic diversity exhibited by CCHFV strains results in significant antigenic differences that will need to be taken into consideration for vaccine development.

Mapping of a neutralizing epitope. Since neutralizing MAb 11E7 was able to recognize G_C by Western blot under non-reducing conditions, we were able to partially map its epitope by testing its ability to recognize fragments of G_C produced in 293T cells. This is of relevance because MAb 11E7 protects mice in vivo from challenge with CCHFV strain IbAr10200 (1). Passive immunization can be effective for the treatment of CCHFV infection in humans, emphasizing the importance of identification of neutralizing antibodies and the epitopes to which they bind (6). The neutralizing epitope of MAb 11E7 is contained between amino acids 1443 and 1566 of the M segment of IbAr10200 strain, a highly conserved region of the protein.

Task 2. Identify cell receptors and attachment factors for CCHFV.

Although we initially planned to develop this part of the project during the second year, we have started to produce tools that will allow us to study CCHFV entry in BSL2 facilities.

CCHFV M segment codon optimization. The CCHFV glycoproteins accumulate in the Golgi, the site of virus budding. The signal for Golgi localization is present in the G_N glycoprotein, specifically within the ectodomain. The clustering of the glycoproteins in the Golgi represents an obstacle for the study of virus entry outside of BSL-4 containment. Indeed, fusion assays, pseudovirion or virus-like particle production, some of the techniques to rapidly study and characterize viral entry in BSL-2 facilities, require expression of the glycoproteins on the cell surface. Golgi retention mechanisms are saturable and therefore overexpression of the glycoproteins may result in mobilization of the glycoproteins to the cell surface. Codon optimization consists of the modification of the codons of a gene without altering the amino acid sequence in order to utilize only the most abundant codons present in each determined species. To increase CCHFV G_N and G_C expression and consequently to drive the proteins to the cell surface, we produced a codon optimized version of the CCHFV M segment. We analyzed the expression and intracellular localization of the CCHFV glycoproteins utilizing monoclonal antibodies (MAbs) to both the G_N and G_C proteins. Unfortunately, there is not an evident difference between processing and localization of the glycoproteins produced by the native gene and the ones produced by the codon optimized gene. This suggests that properties intrinsic of the protein structure and processing may be blocking the transport of the glycoproteins to the surface.

CCHFV fusion assay. CCHFV must be handled in BSL-3 or BSL-4 facilities; however, a CCHFV cell-cell fusion assay facilitates its study by allowing investigation of CCHFV glycoprotein function under BSL-2 conditions. As for other members of the *Bunyaviridae* family, CCHFV glycoproteins are targeted to the Golgi, where the virus buds. The glycoproteins are typically not detected on the cell surface, preventing cell-cell fusion. We codon optimized the CCHFV glycoproteins and found that over-expression of CCHFV glycoproteins using the codon optimized vector resulted in the expression of the glycoproteins on the surface of the cell. Using this system, we developed a cell-cell fusion assay. Brief treatment of CCHFV glycoprotein expressing cells with mildly acid media (pH 5.9 and below) resulted in rapid and efficient syncytia formation. Fusion was quantified using β -galactosidase complementation, based on the fact that β -galactosidase functions as a tetramer and the alpha and omega subunits of β -galactosidase can be transfected individually into cells. Fusion driven by the expression of CCHFV glycoproteins on the cell surface after acid treatment drive syncytia formation, allowing the alpha and omega subunits to form the active β -galactosidase tetrameric enzyme. The activity of β -galactosidase can then be measured on a luminometer. We have shown the CCHFV fusion is pH dependent using this assay, and the fusion activity can be inhibited using antibodies directed against the glycoproteins of CCHFV. Such an assay will make it possible to rapidly study the membrane fusion activity of CCHFV glycoproteins, and to

screen antibodies and small molecule inhibitors for the ability to inhibit virus specific membrane fusion.

3. Key research accomplishments.

Our key research accomplishments are listed under **Conclusions** (Section 5) and in the two papers listed under **Reportable Outcomes** (Section 4).

4. Reportable outcomes.

Two papers and five abstracts were submitted during the first year of this project, with both papers being accepted for publication. These are listed below.

PAPERS

Bertolotti-Ciarlet, A., Smith J., Strecker K., Paragas J., Altamura L., McFalls J., Frias-Staheli N., García-Sastre, A., Schmaljohn, C., and Doms, R.W. (2005) Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. *J.Virol.* 79: 6152-61.

Ahmed, A., McFalls, J., Hoffmann, C., Filone, C.M., Stewart S. M., Paragas, J., Khodjaev S., Shermukhamedova D., Schmaljohn, C.S., Doms, R.W., and Bertolotti-Ciarlet, A. (2005) Presence of broadly reactive and group-specific neutralizing epitopes on newly described isolates of Crimean-Congo hemorrhagic fever virus. *Journal of General Virology*. In Press.

ABSTRACTS TO MEETINGS

1. Bertolotti-Ciarlet, A., Smith J., Strecker K., Altamura L., Paragas J., García-Sastre A., Frias-Staheli N., Schmaljohn C., and Doms RW. *Cellular processing and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins*. Bioterrorism and Emerging Infectious Diseases: Antimicrobial, Therapeutics and Immune-Modulators. January 6-11, 2004. Keystone resort, Keystone Colorado, U.S.
2. Bertolotti-Ciarlet, A., Smith J., Strecker K., Altamura L., Paragas J., García-Sastre A., Frias-Staheli N., Schmaljohn C., and Doms RW. *Cellular localization and antigenicity of Crimean-Congo hemorrhagic fever virus glycoproteins*. The 23rd Annual Meeting of the American Society for Virology. July 10-14, 2004. McGill University, Montreal, Canada.
3. McFalls J., Smith J., Bertolotti-Ciarlet A., Strecker K., Paragas J., Schmaljohn C., and Doms RW. *Mapping a neutralizing epitope on Crimean-Congo hemorrhagic fever virus glycoprotein G_C*. The 23rd Annual Meeting of the American Society for Virology. July 10-14, 2004. McGill University, Montreal, Canada.
4. L. Altamura, A. Bertolotti-Ciarlet, J. McFalls, J. Paragas, C. Schmaljohn, and R. W. Doms. *Oligomeric Structure of Crimean-Congo Hemorrhagic Fever Virus Glycoproteins*. The 24th Annual Meeting of the American Society for Virology. June 18-22, 2005; The Pennsylvania State University, State College, PA.
5. N. Frias-Staheli, J. Paragas, C. Schmaljohn, and A. García-Sastre. *Clonaje, secuenciación y caracterización de la RNA polimerasa dependiente de RNA de CCHFV*. VII Congreso Argentino de Virología. September 19-22. Buenos Aires, Argentina.

5. Conclusions.

The major conclusions reached during the first year of this project are as follows:

1. IBR 10-200 is growth restricted in human macrophages unlike 3010.
2. Cell cytokine levels have been determined in response to viral infection in a cell system
3. Intact virus support was provided to projects 1 and 2. A graduate student was fully trained to work at USMRIID in the high biocontainment laboratories.
4. The sequence of the L protein of CCHFV has been obtained
5. The L protein of CCHFV is a cytoplasmic protein with an apparent molecular weight of 450kDa.
6. A luciferase reporter gene construct flanked by the 3' and 5' UTR of the S segment of CCHFV is replicated and transcribed in CCHFV-infected cells.
7. The N protein of CCHFV has RNA-binding activity.
8. The GN and GC proteins are targeted to the Golgi when expressed together. When expressed alone, GN is transported to the Golgi while GC is retained in the ER.
9. Codon optimization of the CCHFV M segment makes it possible to over-express the GN and GC glycoproteins, resulting in the delivery of some of the proteins to the cell surface.
10. The GN and GC proteins present on the cell surface can mediate cell-cell fusion at acid pH. This makes it possible to study the function of these proteins under BSL2 conditions.
11. The GC protein forms a dimer.
12. There is significant antigenic variability between different CCHFV strains isolated from different parts of the world. While this has obvious implications for vaccine development, it is important to recognize that some neutralizing epitopes are highly conserved, making these important vaccine targets.

6. References.

1. **Bertolotti-Ciarlet, A., J. Smith, K. Strecker, J. Paragas, L. A. Altamura, J. M. McFalls, N. Frias-Staheli, A. García-Sastre, C. S. Schmaljohn, and R. W. Doms.** 2005. Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. *J. Virol.* **79**:6152-6161.
2. **Flick, R., K. Flick, H. Feldmann, and F. Elgh.** 2003. Reverse genetics for Crimean-Congo hemorrhagic fever virus. *J. Virol.* **77**:5997-6006.
3. **Honig, J. E., J. C. Osborne, and S. T. Nichol.** 2004. Crimean-Congo hemorrhagic fever virus genome L RNA segment and encoded protein. *Virology* **321**:29-35.
4. **Kinsella, E., S. G. Martin, A. Grolla, M. Czub, H. Feldmann, and R. Flick.** 2004. Sequence determination of the Crimean-Congo hemorrhagic fever virus L segment. *Virology* **321**:23-28.
5. **Sanchez, A. J., M. J. Vincent, and S. T. Nichol.** 2002. Characterization of the glycoproteins of Crimean-Congo hemorrhagic fever virus. *J. Virol.* **76**:7263-7275.
6. **Vassilenko, S. M., T. L. Vassilev, L. G. Bozadjiev, I. L. Bineva, and G. Z. Kazarov.** 1990. Specific intravenous immunoglobulin for Crimean-Congo haemorrhagic fever. *Lancet* **335**:791-792.
7. **Vincent, M. J., A. J. Sanchez, B. R. Erickson, A. Basak, M. Chretien, N. G. Seidah, and S. T. Nichol.** 2003. Crimean-Congo hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1. *J. Virol.* **77**:8640-8649.

7. Appendices.

Two papers are included as Appendix materials:

Bertolotti-Ciarlet, A., Smith J., Strecker K., Paragas J., Altamura L., McFalls J., Frias-Staheli N., García-Sastre, A., Schmaljohn, C., and Doms, R.W. (2005) Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. *J. Virol.* 79: 6152-61.

Ahmed, A., McFalls, J., Hoffmann, C., Filone, C.M., Stewart S. M., Paragas, J., Khodjaev S., Shermukhamedova D., Schmaljohn, C.S., Doms, R.W., and Bertolotti-Ciarlet, A. (2005) Presence of broadly reactive and group-specific neutralizing epitopes on newly described isolates of Crimean-Congo hemorrhagic fever virus. *Journal of General Virology*. In Press.

Cellular Localization and Antigenic Characterization of Crimean-Congo Hemorrhagic Fever Virus Glycoproteins

Andrea Bertolotti-Ciarlet,¹ Jonathan Smith,² Karin Strecker,¹ Jason Paragas,³
Louis A. Altamura,¹ Jeanne M. McFalls,¹ Natalia Frias-Stäheli,⁴
Adolfo García-Sastre,⁴ Connie S. Schmaljohn,³
and Robert W. Doms^{1*}

Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania 19104¹;
AlphaVax, Inc., 2 Triangle Drive, Research Triangle Park, North Carolina 27709²;
Virology Division, U.S. Army Medical Research Institute for Infectious Diseases,
Fort Detrick, Frederick, Maryland 21702³; and Department of Microbiology,
Mount Sinai School of Medicine, One Gustav L. Levy Place,
New York, New York 10029⁴

Received 30 July 2004/Accepted 21 December 2004

Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus *Nairovirus* of the family *Bunyaviridae*, causes severe disease with high rates of mortality in humans. The CCHFV M RNA segment encodes the virus glycoproteins G_N and G_C. To understand the processing and intracellular localization of the CCHFV glycoproteins as well as their neutralization and protection determinants, we produced and characterized monoclonal antibodies (MAbs) specific for both G_N and G_C. Using these MAbs, we found that G_N predominantly colocalized with a Golgi marker when expressed alone or with G_C, while G_C was transported to the Golgi apparatus only in the presence of G_N. Both proteins remained endo- β -N-acetylglucosaminidase H sensitive, indicating that the CCHFV glycoproteins are most likely targeted to the *cis* Golgi apparatus. Golgi targeting information partly resides within the G_N ectodomain, because a soluble version of G_N lacking its transmembrane and cytoplasmic domains also localized to the Golgi apparatus. Coexpression of soluble versions of G_N and G_C also resulted in localization of soluble G_C to the Golgi apparatus, indicating that the ectodomains of these proteins are sufficient for the interactions needed for Golgi targeting. Finally, the mucin-like and P35 domains, located at the N terminus of the G_N precursor protein and removed posttranslationally by endoproteolysis, were required for Golgi targeting of G_N when it was expressed alone but were dispensable when G_C was coexpressed. In neutralization assays on SW-13 cells, MAbs to G_C, but not to G_N, prevented CCHFV infection. However, only a subset of G_C MAbs protected mice in passive-immunization experiments, while some nonneutralizing G_N MAbs efficiently protected animals from a lethal CCHFV challenge. Thus, neutralization of CCHFV likely depends not only on the properties of the antibody, but on host cell factors as well. In addition, nonneutralizing antibody-dependent mechanisms, such as antibody-dependent cell-mediated cytotoxicity, may be involved in the *in vivo* protection seen with the MAbs to G_C.

Crimean-Congo hemorrhagic fever virus (CCHFV) causes a hemorrhagic and toxic syndrome in humans with mortality rates of up to 50%. CCHFV infection was first described during an outbreak in Russia during the 1940s, when more than 200 cases of severe hemorrhagic fever were reported among agricultural workers and soldiers in the Crimean peninsula (15, 16). Since then, the virus has spread throughout many regions of the world, including sub-Saharan Africa (60, 61), Bulgaria, the Arabian Peninsula, Iraq, Pakistan, the former Yugoslavia, northern Greece, and northwest China (16, 23, 42–45).

CCHFV is a member of the genus *Nairovirus* within the family *Bunyaviridae* (52). Members of this enveloped-virus family have a tripartite, single-stranded RNA genome of negative polarity. The medium RNA segment (the M segment)

encodes the viral glycoproteins G_N and G_C, which, like those of other *Bunyaviridae*, are synthesized as polypeptide precursors that undergo proteolytic cleavage events to yield mature glycoproteins (52). The CCHFV glycoproteins exhibit several unusual structural features and undergo several processing events. First, the CCHFV glycoproteins contain, on average, 78 to 80 cysteine residues, suggesting the presence of an exceptionally large number of disulfide bonds and a complex secondary structure. Second, the G_N precursor protein (Pre-G_N) contains a highly variable domain at its amino terminus that contains a high proportion of serine, threonine, and proline residues, and it is predicted to be heavily O glycosylated, thus resembling a mucin-like domain present in other viral glycoproteins, most notably the Ebola virus glycoprotein (56). The mucin-like region in the Ebola glycoprotein has been shown to play an important role in a cell-rounding phenotype and immunoevasion (39, 56). It is not known whether this domain plays an important role in CCHFV pathogenesis or whether it is even O glycosylated. A third unusual feature is

* Corresponding author. Mailing address: Department of Microbiology, University of Pennsylvania, 225 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104. Phone: (215) 898-0890. Fax: (215) 898-9557. E-mail: doms@mail.med.upenn.edu.

that the G_N glycoprotein can undergo two posttranslational proteolytic cleavage events at the conserved motifs RSKR and RRL, potentially releasing the mucin-like domain as well as a second N-terminal domain of approximately 35 kDa (P35, or the connector domain) (59). It is not known if the released domains traffic to an intracellular compartment, if they are secreted, or what effect they may have on viral pathogenesis and antigenic structure. Similar processing strategies have not been observed for other *Bunyaviridae* outside of the *Nairovirus* group.

As the only virally encoded membrane proteins, G_N and G_C must interact with cell surface receptors, mediate the entry of virus into cells, and serve as targets for neutralizing antibodies. Passive transfer of neutralizing antibodies can protect susceptible animals from hantavirus infection (8, 53–55, 62), and there is a report that convalescent-human sera can afford some protection in acutely infected individuals (58). Thus, characterizing the structures and functions of these proteins will be important for understanding CCHFV tropism and pathogenesis as well as for vaccine development. In this study, we describe the first monoclonal antibodies (MAbs) raised against the CCHFV glycoproteins, map the subunits to which they bind, and characterize their abilities to neutralize virus and to protect mice from a lethal CCHFV challenge. In addition, using these MAbs, we investigated the localization of G_N and G_C when expressed alone or together and have begun to map the regions involved in glycoprotein localization and interactions.

MATERIALS AND METHODS

Cells, antibodies, and viruses. CCHFV prototype strain IbAr10200, first isolated in 1976 from *Hyalomma excavatum* ticks from Sokoto, Nigeria, was grown in African green monkey kidney Vero cells or the E6 variant (51). African green monkey kidney fibroblast (CV-1), Vero, Vero E6, human cervix carcinoma (HeLa), and human embryonic kidney (HEK 293T) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Similarly, the human tumor cell line SW-13 (adrenocortical carcinoma) was grown in DMEM supplemented with 2.5% fetal bovine serum. Work with CCHFV was performed in a biosafety level 4 laboratory at the U.S. Army Medical Research Institute for Infectious Diseases. A recombinant vaccinia virus expressing the T7 bacteriophage RNA polymerase (vTF1.1) was grown in HeLa cells, and titers were determined in CV-1 cells according to standard protocols (1).

Production of MAbs. MAbs were prepared against the G_N and G_C glycoproteins of the CCHFV strain IbAr10200 by fusion of SP2/0 myeloma cells with splenocytes from BALB/c inbred mice. We carried out five independent fusions in which mice were immunized with infected suckling mouse brain homogenates (fusions I and II; MAb 30F7), with affinity-purified virion glycoproteins precipitated from nonionic detergent lysates of gradient-purified virus preparations (fusions III and IV; MAbs 1H6, 5E3, 6C2, 5B5, 8C4, 9H3, 3E3, 8G7, 8A8, and 8F10), or with affinity-purified proteins from similar lysates of infected cells (fusion V; MAbs 8A1, 12A9, 11E7, 6C11, 13G8, 14B6, 10G4, 5A5, 6B12, 10E11, 3F3, 2C9, 7F5, 11F6, and 9C6). In earlier studies, we found that hybridoma fusions carried out with splenocytes from mice immunized with mouse brain homogenates resulted predominantly in MAbs that were reactive with the viral nucleocapsid protein. To produce immunogens enriched in viral glycoproteins, cultures of SW-13 cells in 34 T-150 flasks were infected with the IbAr10200 strain of CCHFV and incubated for 36 h at 37°C. Medium fractions were removed, clarified at 10,000 × g, and centrifuged in an SW28 rotor (Beckman) to pellet virus particles (25,000 rpm for 3 h). Virus pellets were lysed in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 2% Triton X-100, 1% sodium deoxycholate, and protease inhibitors) and subsequently centrifuged to equilibrium in CsCl gradients. This resulted in a separation of the viral nucleocapsid (density = 1.3 g/ml) from the viral glycoproteins which banded in a broad band at lower densities. Infected-cell monolayers were similarly lysed in immunoprecipitation buffer, clarified at 10,000 × g, and centrifuged in an

SW41 rotor (Beckman) to sediment the viral nucleocapsid from the solubilized viral glycoproteins (40,000 rpm for 4 h). Fractions enriched for viral glycoproteins from both cell and virion samples were then collected on solid-phase immunoadsorbents prepared by saturating protein A-Sepharose with anti-CCHFV hyperimmune mouse ascitic fluid (D. Watts, U.S. Army Medical Research Institute for Infectious Diseases). Immunoadsorbents were then exhaustively washed with phosphate-buffered saline (PBS), emulsified directly in complete or incomplete Freund's adjuvant, and inoculated subcutaneously and intraperitoneally into mice as described below. With this procedure, the CCHFV glycoproteins were enriched but were not pure. Importantly, the viral nucleocapsid was not detectable under these conditions, and using these immunogens, we were able to prepare a large number of CCHFV glycoprotein-specific MAbs.

MAbs were produced essentially as described previously (28). Briefly, BALB/c mice were twice immunized intraperitoneally with 100 µl of antigen preparations emulsified in Freund's complete adjuvant for primary immunization and in Freund's incomplete adjuvant for secondary immunization. Mice were euthanized 3 to 5 days after a third immunization, and splenocytes were fused with Sp2/0-Ag14 myeloma cells. Hybridoma cultures were incubated at 37°C with several changes of hypoxanthine-aminopterin-thymidine medium, and the supernatant fluids were screened by immunofluorescence (IF) assays against CCHFV-infected Vero cells and by enzyme-linked immunosorbent assays (ELISA) with viral antigen from suckling mouse brain homogenates or gradient-purified virus preparations. Antigenic specificity was initially determined by immunoprecipitation from [³⁵S]methionine-labeled infected-cell lysates (as described below) and subsequently by IF microscopy. Positive-antibody-producing cells were cloned by limiting dilution and then expanded. The immunoglobulin G subclasses of the resulting MAbs were determined by indirect ELISA analysis using hybridoma supernatants. The ELISA was developed using immunoglobulin G subclass-specific immunoglobulins (Miles Laboratories) by following the manufacturer's instructions.

Neutralization assays. Eighty percent plaque reduction neutralization (PRN-80) tests were carried out on SW-13 cell monolayers. Twofold serial dilutions of the MAbs were mixed with 200 PFU of the CCHFV IbAr10200 strain and incubated for 1 h at 37°C. Confluent monolayers of SW-13 cells in six-well plates were incubated with the virus-antibody mixture for 1 h at 37°C. The inocula were removed, and 1 ml of overlay consisting of 1 part double-strength DMEM with 5% fetal bovine serum and 1 part low-gelling-temperature agarose (Bio-Rad Laboratories, Richmond, CA) in distilled water was added. After incubation at 35°C in a sealed chamber for 2 to 5 days, the plaques were visualized by neutral red staining.

Protection studies. To evaluate the protective activities of MAbs directed against the CCHFV glycoproteins in an animal model, suckling mice, which are susceptible to infection with CCHFV (24, 50), were challenged with live virus before or after passive immunization with the CCHFV-specific MAbs. Two- to 3-day-old suckling mice were inoculated in groups of five to eight by intraperitoneal injection with 50 µl of undiluted ascitic fluid containing the different MAbs. The ascitic fluids were administered 24 h before or after the inoculation of 100 50% lethal-dose units of the CCHFV strain IbAr10200. Ascitic fluid from Sp2/0 cells that did not contain virus-specific antibodies was used as a negative control.

Construction of CCHFV glycoprotein clones. The pCAGGS-M clone was created by cloning the entire M segment of IbAr10200 into the NruI and XhoI sites found in the plasmid pCAGGS-MCSII (41). The M segment was digested using the unique restriction enzyme sites SnaBI and SalI located in the untranslated regions of the gene. This clone was used as a template for the generation of a panel of constructs used to map functional regions on CCHFV glycoproteins (Fig. 1). Primers were synthesized according to the published sequence for IbAr10200 (51), and standard PCR technology was performed for cloning into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). The 5'-end primers included the CACC sequence at the 5' end and the start codon to allow for directional cloning. The 3'-end primers did not possess a stop codon to allow the inclusion of the V5 and His epitope tags at the C terminus of the protein. The cloning was performed as described by the manufacturer (Invitrogen), and all constructs were sequenced. All primer sequences are available upon request.

Protein analysis. To analyze protein expression, we transfected HEK 293T cells using Lipofectamine 2000 (Invitrogen). After 24 h, cell extracts were prepared in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, and Complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN). Cell lysates were incubated at 4°C for 3 min and then centrifuged at 10,000 × g for 10 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 4 to 15% Tris-HCl gels (Bio-Rad, Hercules, CA), followed by Western blot analysis with mouse anti-V5 (Invitrogen) as the primary antibody and sheep anti-mouse horseradish peroxidase conjugate as the second-

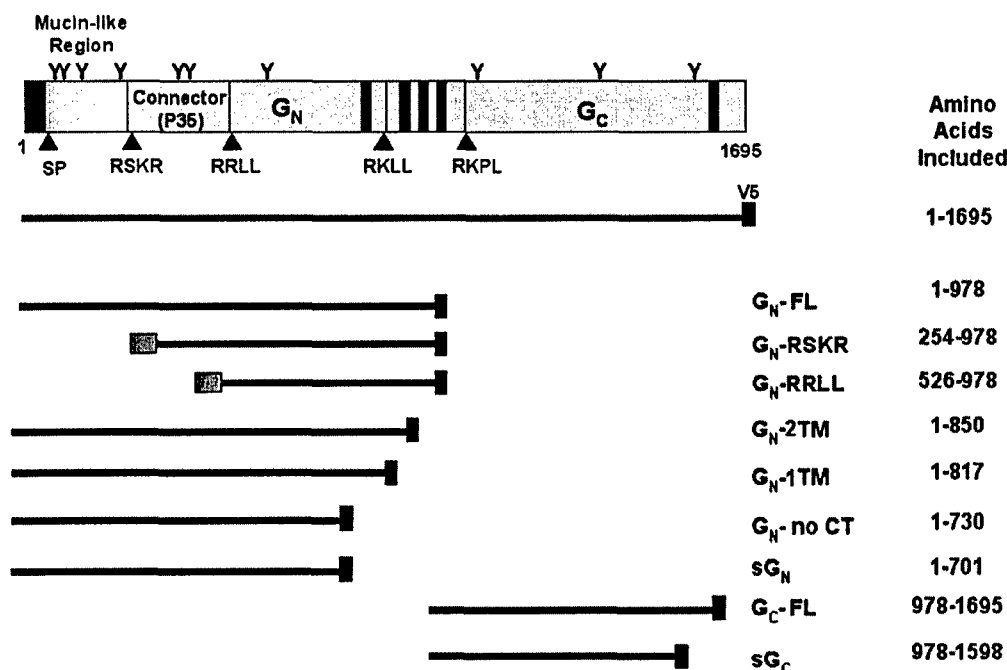


FIG. 1. Schematic representation of CCHFV M segment constructs. Predicted N-linked glycosylation sites are indicated with "Y." The filled triangles (\blacktriangle) indicate the predicted conserved cleavage sites with the four conserved amino acid cleavage sites indicated (59), and the black bars indicate the predicted TMs. Specific constructs used in this study are also indicated by black lines, indicating which portions of the CCHFV M segment are included in each construct. The gray boxes on the constructs G_N -RSKR and G_N -RRLL indicate the inclusion of the hen egg white lysozyme signal peptide at the N terminus of the protein (6). A V5 epitope tag was positioned at the C terminus of each construct; these tags are represented as black boxes on the far-right side of each construct. Finally, the amino acids included in each construct are indicated on the right column, using the first methionine in the IbAr10200 strain as residue 1.

ary antibody (Amersham Pharmacia, Buckinghamshire, United Kingdom), followed by visualization with ECL-Plus Western blotting detection reagents (Bioscience, Piscataway, NJ).

IF microscopy. Localization of the CCHFV glycoproteins by indirect IF was performed as described previously (38). HeLa cells grown to 50% confluence on glass coverslips were infected with the recombinant vaccinia virus vTF1.1 (1), followed 40 min later by transfection with the different pcDNA3.1/V5-His constructs (Fig. 1). When proteins were expressed with pCAGGS constructs, vTF1.1 infection was not utilized, since protein expression was obtained from the chicken beta-actin promoter. At 24 h posttransfection, the cells were fixed with 2% (wt/vol) paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100, and stained with a G_N - or G_C -specific MAb ascites fluid (diluted 1:250) or with mouse anti-V5 MAb (diluted 1:500) (Invitrogen) in PBS containing 0.5 mM $MgCl_2$ and 4% fetal bovine serum. In addition, TGN46, a sheep antibody specific for a heavily glycosylated protein localized primarily in the trans-Golgi network, was included as a marker for Golgi localization (Serotec, Oxford, United Kingdom). Then, cells were washed with PBS and incubated for 1 h with the secondary antibodies conjugated to Alexa Fluor 488 (goat anti-mouse) and Alexa Fluor 594 (goat anti-sheep) (Molecular Probes, Eugene, OR) diluted 1:500 in PBS-4% fetal bovine serum. Finally, cells were washed in PBS, mounted in Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), and examined on a Nikon E600 microscope at a magnification of $\times 60$ utilizing UV illumination.

Endoglycosidase treatment. The method of Trimble and Maley (57) was slightly modified for digesting glycosylated CCHFV glycoproteins with endo- β -N-acetylglucosaminidase H (endo-H). Cell lysates from transfected HEK 293T cells were boiled for 5 min in a 50 mM sodium citrate buffer (pH 5.5) containing 1% SDS and 200 μ g/ml of phenylmethylsulfonyl fluoride. After cooling, the samples were supplemented with an equal volume of 0.02 U of endo-H (New England Biolabs Inc., Beverly, MA) in a 50 mM sodium citrate buffer (pH 5.5) or the same buffer without the enzyme before incubation at 37°C for 20 h. After this incubation, the samples were analyzed by SDS-PAGE. In addition, the glycoproteins were treated with peptide-N-glycosidase F (PNGase F) (New England Biolabs). A similar protocol was followed for PNGase F treatment, but the digestion was performed in 100 mM sodium phosphate, pH 7.5, with 0.75% NP-40, 0.1% SDS, and 50 mM β -mercaptoethanol.

Immunoprecipitations from infected cells. Immunoprecipitation assays were performed as previously described (38). Briefly, SW-13 cell monolayers (1×10^6 cells) were infected with the CCHFV strain IbAr10200 at a multiplicity of infection of 5. Infected SW-13 cells were placed in methionine-free DMEM at 28 h postinfection for 30 min, and then 30 μ Ci of [35 S]methionine (Trans- 35 S-label; ICN, Irvine, CA) was added. After 1 h of labeling, 50 μ g of unlabeled methionine per ml was added. Infected cells were collected 24 h postlabeling or 20 h posttransfection and lysed with 0.5 ml of lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% CHAPSO {3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate}, 5 mM EDTA, and a Complete protease inhibitor mixture (Roche). Precleared lysates were incubated for 1 h at 4°C with the MABs. Glycoproteins were immunoprecipitated using 7 μ g of the MABs generated in this study. The MABs were previously coupled to protein A/G Plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitated proteins were eluted at 100°C for 5 min with 20 μ l of sample buffer (0.08 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.005% bromophenol blue). The immunoprecipitated proteins were detected after separation by 10% SDS-PAGE utilizing a personal densitometer (model 860; Molecular Dynamics, Inc., Sunnyvale, CA), and the data were analyzed with the ImageQuant NT software.

Immunoprecipitation from transfected cells. For the analysis of the different CCHFV glycoprotein constructs, HEK 293T cell monolayers (1×10^6) were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The immunoprecipitation protocol utilized was in general the same as that described above for infected cells. The V5 epitope tag present at the C terminus of the recombinant proteins was detected using 7 μ g of a MAB directed against the V5 tag (Invitrogen). Samples from transfected cells were analyzed by Western blotting as described above, using a rabbit anti-V5 tag hyperimmune serum diluted 1:5,000 (Sigma).

RESULTS

CCHFV M segment processing. Glycoproteins of the nairovirus group of bunyaviruses are processed in a distinctive and

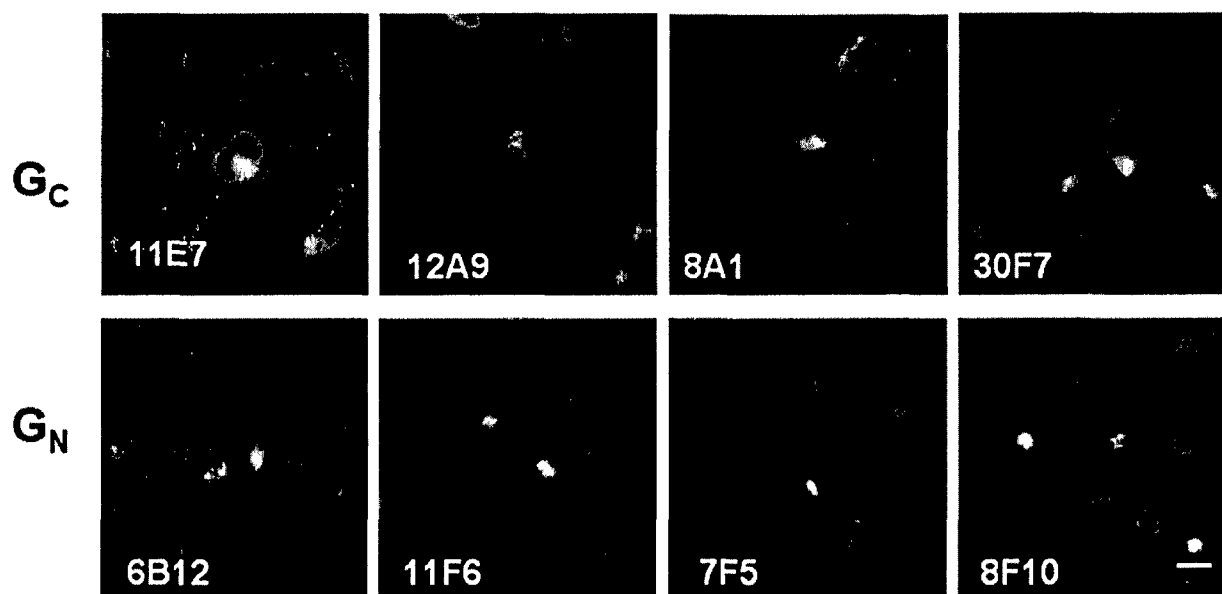


FIG. 2. Analysis of G_N and G_C cellular localization. HeLa cells were transfected with a pCAGGS vector expressing the full-length M segment of CCHFV. After 24 h, the cells were fixed and stained using the indicated MABs in addition to an antibody specific for the Golgi resident protein TGN46 as described in Materials and Methods. The images show the Golgi apparatus (red) and the glycoproteins (green) merged. Yellow indicates colocalization. The secondary antibodies used were Alexa Fluor 488 (goat anti-mouse) and Alexa Fluor 594 (goat anti-sheep). Magnification, $\times 60$; bar = 100 nm.

complex manner (51, 59). The M segment of CCHFV encodes a polyprotein that undergoes proteolytic processing to yield a 140-kDa Pre- G_N (termed G_N -FL in Fig. 1) and an 85-kDa precursor of G_C (Pre- G_C ; termed G_C -FL in Fig. 1). Recently, it was shown that the N terminus of the mature G_N protein contains both a mucin-like and a P35 connector domain (Fig. 1) that are removed by specific cleavage with protease SKI-1, resulting in the generation of mature G_N (G_N -RRLL in Fig. 1) (59). However, how G_N and G_C are cleaved from each other is not clear, though it is thought that the N terminus of the mature G_C protein is generated by cleavage at a conserved RKPL site (59). Finally, cleavage between the mucin domain and the P35 region may be mediated by furin, which is consistent with the presence of a conserved RSKR sequence between these domains (Fig. 1) (59).

MABs to CCHFV. To assist in our studies of CCHFV glycoprotein processing and localization, and to determine if antibodies can confer protection to CCHFV, we produced a panel of MABs from mice immunized with a variety of CCHFV antigens as described in Materials and Methods. MABs to CCHFV were identified by ELISA, after which both immunoprecipitation and immunofluorescence studies were done to identify the glycoprotein subunit to which each MAB bound. Only the 26 MABs whose specificity could be clearly identified by both techniques are included in this study. For the immunofluorescence studies, HeLa cells were transfected with pcDNA3.1D/V5-His vectors expressing either G_N (G_N -FL, G_N -RSKR, or G_N -RRLL) or G_C (G_C -FL) and were infected with vTF1.1, a recombinant vaccinia virus that expresses the T7 RNA polymerase (1), in order to achieve high levels of protein expression. The cells were then fixed and processed for IF microscopy. All G_N antibodies recognized G_N -FL but most did

not recognize G_N -RSKR or G_N -RRLL, with the exception of MAB 8F10, which recognized all the G_N constructs. Since neither G_N -RSKR nor G_N -RRLL was correctly transported to the Golgi apparatus (see below), the failure of most G_N MABs to recognize these constructs could be due to protein misfolding rather than the loss of specific epitopes.

From the panel of 26 MABs, we selected six directed against G_N and six directed against G_C for the detailed study of CCHFV glycoprotein processing and targeting. When analyzed by IF and when utilizing HeLa cells transfected with the entire M segment of CCHFV strain IbAr10200, all anti- G_C MABs gave similar patterns, with G_C being localized to both the endoplasmic reticulum (ER) and Golgi regions as judged by colocalization with Golgi and ER markers (Fig. 2 and data not shown). The six anti- G_N MABs recognized G_N only in the Golgi apparatus, suggesting that they recognize epitopes that are either formed after the protein reaches the Golgi apparatus or formed shortly before exit from the ER (Fig. 2). None of the MABs detected G_N or G_C protein on the surfaces of unpermeabilized cells (data not shown). Our results indicate that the CCHFV glycoproteins are not delivered to the cell surface in appreciable quantities, which is consistent with studies that have shown that other *Bunyaviridae* bud into the Golgi apparatus and that their glycoproteins are targeted to this organelle and not delivered to the cell surface (13, 18, 29, 34, 37).

To determine if the MABs could recognize G_N and G_C in different experimental contexts, both Western blot analysis and immunoprecipitation assay were performed. None of the MABs recognized CCHFV proteins under fully denaturing conditions by Western blot analysis. However, in the absence of boiling, MAB 11E7 could recognize G_C obtained from transfected cells or from virus-infected cells by Western blot analysis (Fig. 3).

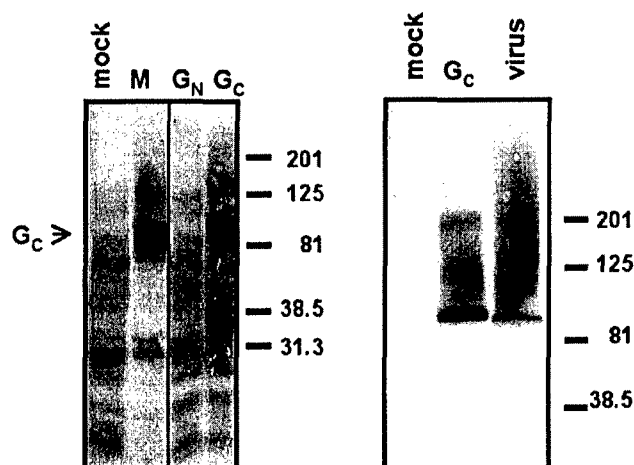


FIG. 3. MAb 11E7 directed against G_C recognizes a linear epitope. HEK 293T cells were transfected with the pCAGGS vector containing the entire CCHFV M segment (M), infected with vaccinia virus vTF1.1 and transfected with the pDNA3.1 constructs containing the G_N or G_C full-length gene with a V5 tag at the C terminus, or mock transfected. Alternatively, SW-13 cells infected with CCHFV were lysed and analyzed by Western blotting (lane labeled "virus"). Western blotting was performed utilizing MAb 11E7. Molecular size markers (in kilodaltons) are noted at the right of each blot.

The failure of most of the MAb to recognize their antigens by Western blot analysis indicates that they bind to conformation-dependent determinants that are lost upon protein denaturation.

The MAbs were also used to immunoprecipitate G_N and G_C from lysates of cells infected with the IbAr10200 strain of CCHFV. All of the MAbs were able to immunoprecipitate the protein subunits to which they bound, which is consistent with their ability to recognize their epitopes by IF. In addition, four of the six G_C MAbs were able to immunoprecipitate both processed G_C (around 75 kDa) and its precursor protein (around 82 kDa), while all of the G_N MAbs analyzed were able to immunoprecipitate processed G_N (35 kDa) and its precursor (140 kDa), which contains both the mucin and the P35 connector domains (data not shown).

Virus neutralization and protection studies. To analyze the in vitro neutralization activities of the panel of MAbs directed against the CCHFV glycoproteins, we performed plaque reduction assays. Twofold serial dilutions of each MAb ascites fluid sample were incubated with 200 PFU of the IbAr10200 strain of CCHFV for 1 h at 37°C prior to addition to confluent SW-13 cells. Plaques were counted 3 to 5 days later, and PRN-80 titers were calculated. None of the MAbs directed against G_N exhibited neutralizing activity in this assay (Fig. 4B), though many of the MAbs directed against G_C neutralized CCHFV in vitro (Fig. 4A).

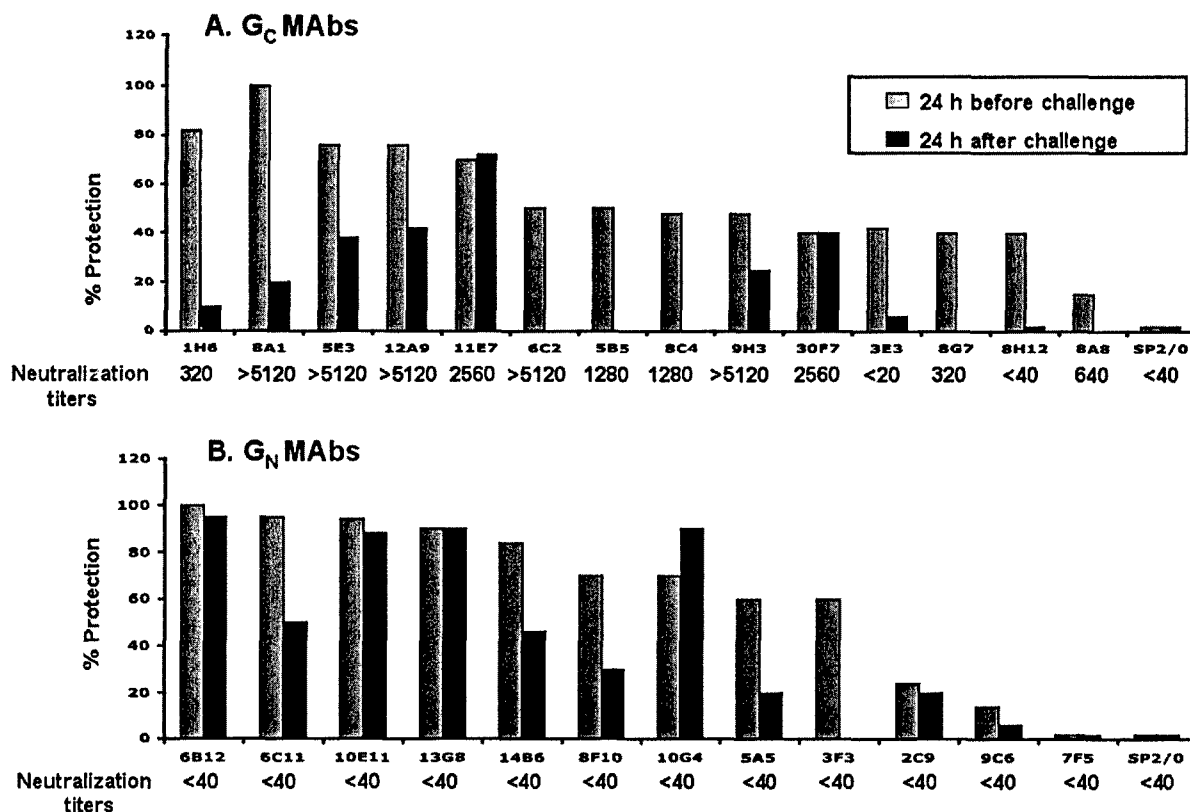


FIG. 4. Correlation between in vitro plaque reduction and in vivo protection. Passive immunization with CCHFV MAb against G_C (A) or against G_N (B). The percentage of protection represents the proportion of animals that survived challenge with respect to the total number of animals treated. Protection was determined in 2- to 3-day-old mice as described in Materials and Methods. The antibodies were provided 24 h before or 24 h after intraperitoneal challenge with the IbAr10200 strain of CCHFV. The plaque reduction neutralization titers are shown at the bottom of each plot for each antibody and represent 80% plaque reduction in SW-13 monolayer cells.

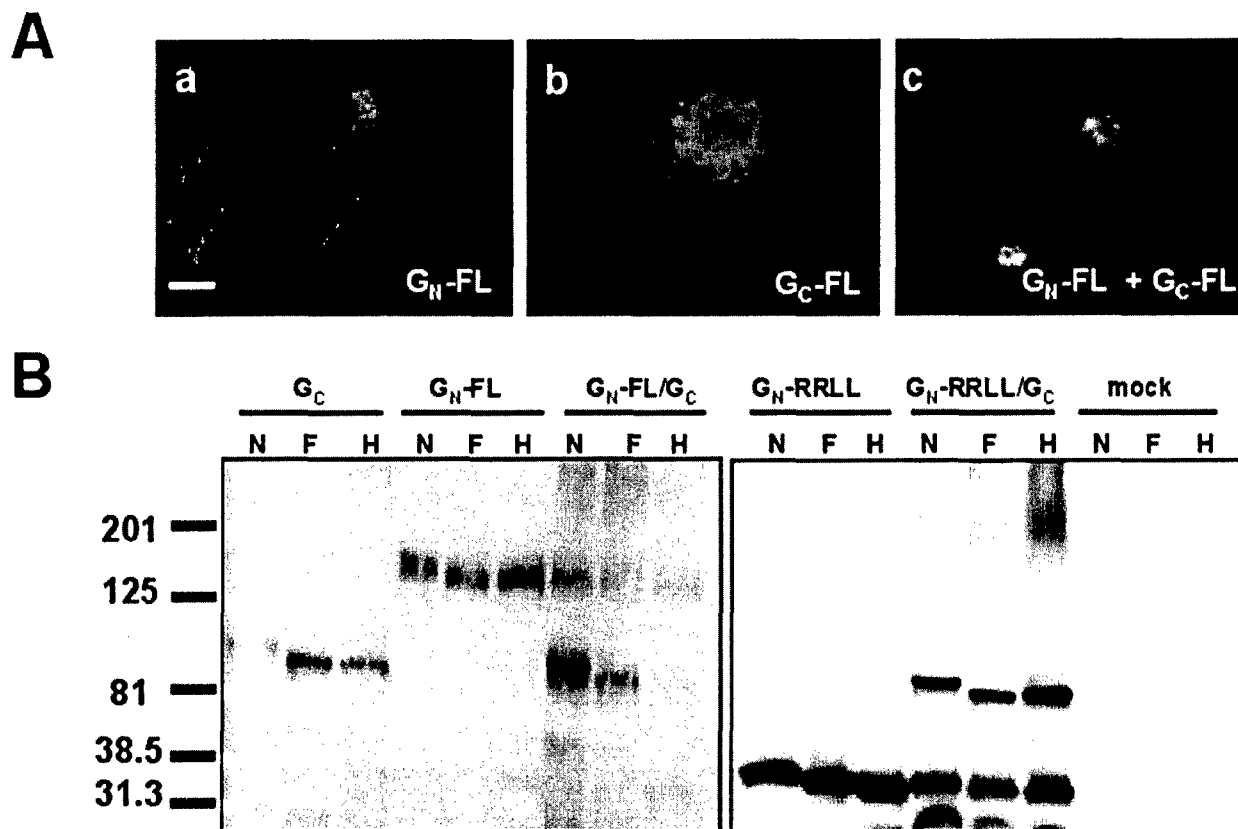


FIG. 5. (A) Localization of CCHFV glycoproteins expressed independently. HeLa cells were infected with recombinant vaccinia virus vTF1.1 and transfected with G_N-FL (a), G_C-FL (b), or both constructs (c). A schematic of the constructs is shown in Fig. 1. Twenty hours posttransfection, the cells were analyzed for the localization of the proteins by IF using MAbs specific for G_N (a) or G_C (b and c). The images show the glycoproteins (in green), the Golgi apparatus (in red), and the extent of colocalization (in yellow). Bar = 100 nm. (B) Endo-H treatment of CCHFV glycoproteins. HEK 293T cells were transfected with the G_N-FL, G_C-FL, or G_N-RRLL constructs separately or together in the presence of recombinant vaccinia virus-expressing T7 polymerase. The lysates were treated with PNGaseF (F) or endo-H (H) or were not treated (N). The lysates were analyzed by Western blotting with mouse anti-V5 antibody. Molecular size markers (in kilodaltons) are noted at the left.

Antibodies have been shown to be effective pre- and post-exposure to prophylaxis treatments for a number of viruses, including cytomegalovirus (9, 10) and respiratory syncytial virus (17, 32). Convalescent-phase serum has been shown to be of benefit to individuals acutely infected with CCHFV (57). Therefore, we tested the CCHFV MAbs for their ability to protect suckling mice challenged with the virus. The MAbs were individually administered by passive immunization to 2- to 3-day-old suckling mice either 24 h before or 24 h after challenge with 100 50% infective-dose units of the IbAr10200 strain of CCHFV (Fig. 4). Protection was registered as the percentage of animals that survived challenge with the live virus (Fig. 4). In general, anti-G_C MAbs that were capable of efficient neutralization *in vitro* protected mice to an appreciable degree when applied before and, to a lesser extent, after virus challenge (Fig. 4). In addition, anti-G_C MAbs that did not neutralize CCHFV infection of SW-13 cells afforded partial protection to mice from CCHFV provided that the MAbs were administered 24 h before viral challenge. When these MAbs were administered 24 h after virus challenge, protection was usually not observed. This suggests that these MAbs do possess some neutralizing activity that was not detected in our *in vitro* assay or that other antibody-based effector mechanisms, such

as antibody-dependent cell-mediated toxicity or complement-mediated cell lysis, function in this context. Likewise, many of the anti-G_N MAbs conferred significant protection to CCHFV challenge, even when applied 24 h after virus challenge and even though they did not prevent virus infection of SW-13 cells *in vitro* (Fig. 4). These results show that there is an imperfect relationship between *in vitro* neutralization and *in vivo* protective ability, at least under the assay conditions used here, and that the ability of an antibody to neutralize CCHFV may depend in part on host factors, as has been observed for La Crosse virus (LACV), another bunyavirus (25–27).

G_N contains a Golgi targeting signal. To analyze the contribution of each CCHFV glycoprotein to Golgi localization, we expressed each protein independently in HeLa cells. IF analyses showed that, while G_N remained in the Golgi apparatus if expressed alone, G_C localized entirely in the ER in the absence of G_N (Fig. 5Aa and -b). When the proteins were expressed together from independent constructs, both G_C and G_N were localized in the Golgi apparatus (Fig. 5Ac). The restoration of G_C Golgi localization in the presence of G_N suggests that G_N possesses a Golgi localization signal and that G_C localizes to the Golgi apparatus through its interaction with G_N (Fig. 5A).

We further analyzed the localization of CCHFV glycoproteins when expressed independently or together by investigating their N-linked glycosylation. The G_N ectodomain of the IbAr10200 strain of CCHFV contains one predicted N-linked glycosylation site, while the G_C ectodomain contains three sites. To determine whether the CCHFV glycoproteins are glycosylated and, if so, modified by medial Golgi enzymes, we used PNGase F, which removes all N-linked carbohydrate chains, or endo-H, which removes immature carbohydrate chains. When G_C -FL was expressed alone, digestion with either endo-H or PNGase F caused the protein to migrate faster (Fig. 5B). When G_N -FL was expressed alone, it too migrated faster following digestion with either endo-H or PNGase F. To determine if the shift observed following glycosidase treatment of G_N -FL was the result of a loss of glycans on the mucin or P35 regions only, we treated the G_N -RRLL protein that lacks both the mucin and the P35 domains with endo-H. The G_N -RRLL protein also migrated faster after endo-H treatment, indicating the presence of N-linked carbohydrate chains on the ectodomain of the mature G_N as well. Similar results were obtained when the proteins were coexpressed (Fig. 5B). Thus, both proteins are N glycosylated, but neither appears to be processed in the medial Golgi apparatus when expressed alone or together.

Contribution of G_N N-terminal domains to protein localization. The CCHFV G_N protein is unusual in that it has two N-terminal domains, a mucin-like domain and the P35 domain, that appear to be cleaved from the G_N precursor in a post-translational fashion (51). The function of these regions is unknown. A similar mucin-like domain has been found to be associated with the glycoprotein of Ebola virus and has been shown to induce cell rounding and detachment in vitro and possibly to be involved in the pathogenicity of the virus (56). To analyze the involvement of the mucin-like domain and P35 region in G_N cellular localization, we deleted these regions from constructs that contained only the G_N portion of the protein in order to generate a construct without the mucin-like domain (G_N -RSKR) and a construct without both the mucin-like domain and the P35 region (G_N -RRLL) (Fig. 1). In both cases, a signal sequence was introduced at the N terminus to ensure proper targeting to the ER. Upon expression in HeLa cells, IF analysis using a MAb to the G_N ectodomain showed that deletion of just the mucin-like domain (G_N -RSKR) resulted in a protein that localized to the Golgi apparatus in a manner similar to full-length G_N -FL (Fig. 6A). In contrast, the G_N -RRLL protein was not present in either the ER or the Golgi apparatus but was distributed in a punctuate pattern that resembled aggresomes, suggesting that it misfolds (Fig. 6B). Thus, the mucin-like domain is dispensable for Golgi targeting, while removal of the P35 connector region affected G_N localization and also led to enhanced degradation. Interestingly, localization of G_N -RRLL to the Golgi complex was recovered when the G_N -RRLL protein was coexpressed with G_C , suggesting that G_N - G_C interactions may promote the correct folding and transport of the proteins (Fig. 6C).

Mapping of the Golgi localization signal. In all *Bunyaviridae* glycoproteins that have been examined, the Golgi localization signal has been localized to G_N , generally in the cytoplasmic tail (CT) or transmembrane domain (TM) of the protein (3–5, 12, 14, 33, 34, 47, 49). The C-terminal domain of G_N contains

a stretch of predicted TMs and cytoplasmic loops between the first amino acid of G_C and the predicted C terminus of G_N (59). The function of this unusual region on glycoprotein processing or any other step of the viral replication and cell cycle is unknown. To analyze the role of this region in G_N localization, we deleted two (G_N 2TM) or three (G_N 1TM) of the predicted four TMs at the G_N C terminus (Fig. 1). HeLa cells were transfected with the constructs and analyzed by IF (Fig. 6). We found that all of the C-terminally truncated G_N constructs localized to the Golgi apparatus (Fig. 6D and E). These results suggest that the Golgi localization signal is not located in this region.

Next, we designed soluble constructs that lacked the transmembrane and cytoplasmic domains of G_N (sG_N) or G_C (sG_C) or that lacked only the CT of G_N (G_N -no CT) (Fig. 1). When these constructs were expressed using the vaccinia virus T7 polymerase system in HeLa cells, both sG_N and G_N -no CT, although also present in the ER, localized to the Golgi apparatus when analyzed by IF microscopy using MAbs against G_N (Fig. 6F and G). In contrast, sG_C was restricted to the ER, just like full-length G_C (Fig. 6H). When sG_C and sG_N were coexpressed, sG_C was then targeted to the Golgi apparatus (Fig. 6I), indicating that the interaction of G_C and G_N occurs through their ectodomains, that the proteins can fold correctly when in their soluble forms, and that Golgi targeting information resides within the ectodomain of G_N .

DISCUSSION

Relatively little is known about the mechanisms by which bunyaviruses enter cells or how infection can be prevented by neutralizing antibodies, and MAbs that block CCHFV infection have not been described. Therefore, we developed a panel of MAbs to assist in our studies on CCHFV glycoprotein biology and to begin characterizing the antigenic structures of G_N and G_C . The large majority of MAbs bound to conformation-dependent epitopes in G_N or G_C . A number of MAbs against G_C , but not against G_N , were able to neutralize virus infection of SW-13 cells in vitro, suggesting that G_C plays an important role in virus entry. Similarly, MAbs directed against the G_C glycoproteins of LACV can inhibit virus infection, with some evidence indicating that this is due to a reduction in virus binding to the cell surface (19, 21, 22, 26). However, some antibodies against G_N can neutralize LACV infection in an insect cell line, though not in a mammalian cell line, suggesting that virus neutralization can be dependent on the cell type being infected and that virus entry mechanisms may differ between invertebrates and vertebrates (30). Consistent with this, proteolytic degradation of G_C with trypsin or pronase virtually eliminates the ability of LACV to bind to vertebrate, but not invertebrate, cell lines (30, 46).

Our results suggest that CCHFV neutralization mechanisms may be complex and context dependent. In general, MAbs directed against G_N were more effective at protecting mice from a lethal CCHFV challenge than were MAbs to G_C when administered either 24 h before or after infection, even though G_N MAbs did not neutralize infection of SW-13 cells in vitro. In addition, not all G_C MAbs that neutralized CCHFV infection in vitro conferred high levels of protection in vivo, especially if administered after infection, as seen for the 8A1

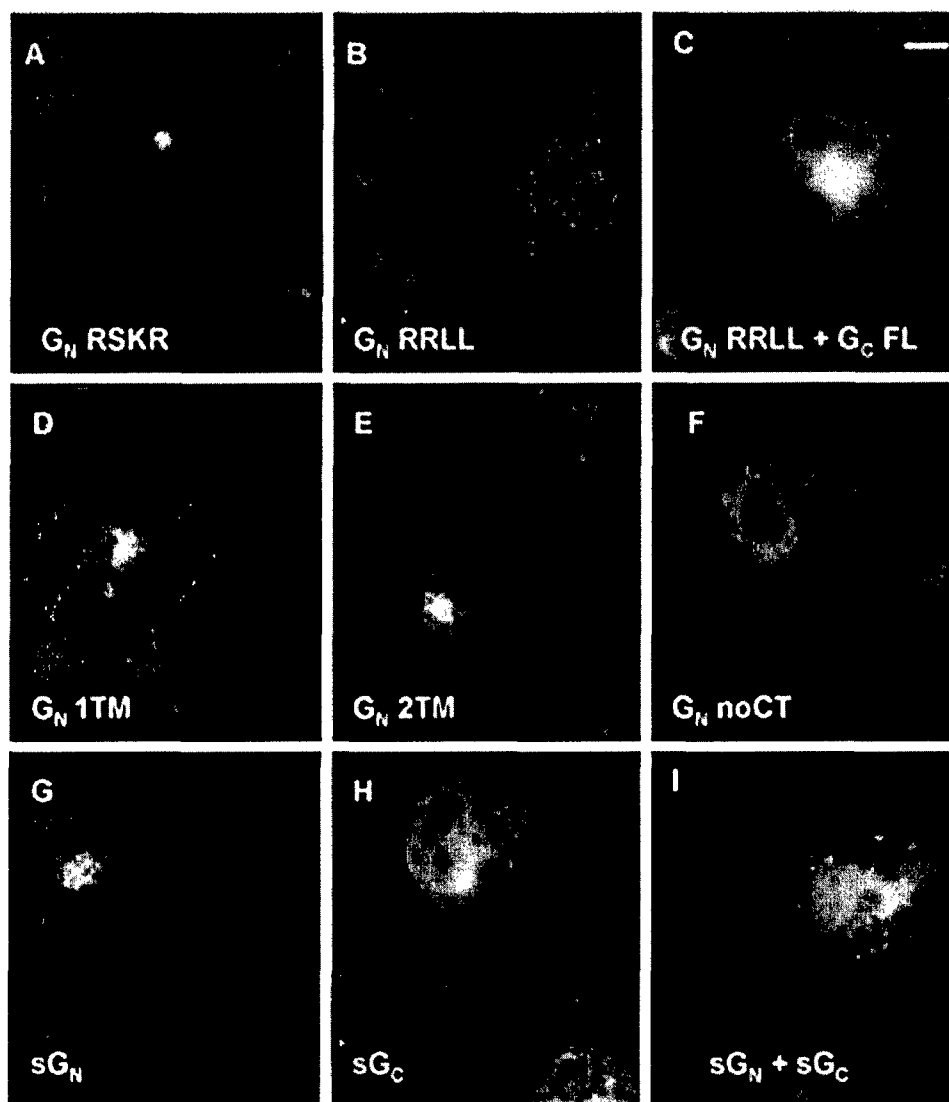


FIG. 6. Immunofluorescence analysis of the CCHFV glycoprotein constructs. HeLa cells were transfected with the indicated CCHFV constructs (shown schematically in Fig. 1) in the presence of recombinant T7 RNA polymerase expressed by vaccinia virus vTF1.1. Twenty hours posttransfection, the cells were fixed and stained using MAb 11E7 against G_C (panels H and I) and MAb 8F10 against G_N (panels A through G) (green) and the TGN46 antibody for Golgi localization (red). Nuclear staining is shown in blue. Bar = 100 nm.

and 1H6 MAbs. Thus, there was not a strict relationship between in vitro neutralization and in vivo protection. As noted above, these results resemble observations on the differential roles of LACV G_N and G_C glycoproteins in viral neutralization and protection. A soluble version of the G_C La Crosse glycoprotein is sufficient to block virus infection in mammalian cells, while antibodies against G_N neutralize infection in a mosquito cell line but not in a vertebrate cell line (31). The mechanisms that account for the differential inhibition of CCHFV infection are not currently clear. We assume that the conformational changes undergone by G_N and G_C to elicit membrane fusion and virus entry will likely be the same regardless of the cell type being infected and that these changes are likely to be induced by acid pH following endocytosis of the virus, as has been documented for La Crosse and Hantaan viruses (7, 20). However, it remains to be determined if CCHFV uses different cell

surface receptors and attachment factors on different cell types. Without knowing the identity of any potential CCHFV receptors, it will be interesting to examine the ability of MAbs to neutralize CCHFV on cell lines derived from different species, including ticks.

One of the hallmarks of the *Bunyaviridae* family is that their glycoproteins are targeted to the Golgi apparatus from which they bud (48, 52). Based on this fact, a number of studies have sought to identify the signals responsible for targeting G_N and G_C to the Golgi apparatus for a number of bunyaviruses (2, 34, 36, 40, 47, 49). Generally, it has been found that the Golgi retention signals reside within the TM and/or CT of the glycoprotein closest to the N terminus of the glycoprotein precursor (18, 34, 35). However, no consensus Golgi localization motif appears to be shared among the glycoproteins of these viruses, and *Nairovirus* glycopro-

tein targeting signals and antigenic structures have not been analyzed so far.

We found that the CCHFV glycoproteins were targeted to the Golgi apparatus, as determined by IF microscopy. Since both G_N and G_C were sensitive to endo-H treatment, it is likely that the proteins are targeted to an early Golgi compartment. Similarly, hantavirus glycoproteins, although present in the Golgi apparatus, remain sensitive to endo-H treatment (49). When G_N and G_C were expressed together, G_N was localized to the Golgi complex while G_C was found in both the Golgi apparatus and the ER. It is obvious that G_N must also be present in the ER, but our conformation-dependent MABs either do not recognize G_N in the ER or bind to epitopes that form just prior to exiting from this organelle. It is also possible that G_N is transported more quickly from the ER than G_C and, at steady state, is below our limit of detection in the ER. Indeed, some studies on the biosynthesis of Uukuniemi virus G_N and G_C proteins showed previously that G_N is transported faster than G_C from the ER to the site of virus budding at the Golgi complex (29). The apparent difference in transport kinetics is due to the fact that G_N folds and is transported from the ER to the Golgi apparatus ~30 to 45 min faster than G_C (4).

The ability of CCHFV G_N to localize to the Golgi apparatus when expressed independently of G_C indicates that G_N contains a Golgi targeting or retention motif. Since G_C is restricted to the ER in the absence of G_N , we conclude that its transport to the Golgi apparatus is dependent upon G_N and likely results from G_N - G_C oligomerization. Whether G_C fails to fold correctly in the absence of G_N , contains an ER retention signal that is masked by G_N association, or lacks a positive transport signal cannot be determined at present. Our results are in agreement with published data about other *Bunyaviridae* glycoproteins (3, 35, 36, 40, 47, 49) which localize to the Golgi apparatus in the absence of any other viral proteins (11, 14, 34, 47). Although for most of the *Bunyaviridae* glycoproteins analyzed to date, the Golgi targeting signal is contained in one of the glycoproteins, for the Hantaan virus, a member of the *Hantavirus* group, both glycoproteins are required to achieve Golgi apparatus targeting (49). Our results with CCHFV indicate that Golgi targeting information resides largely in the ectodomain of the G_N subunit, since a soluble version of G_N was largely restricted to the Golgi apparatus. However, small amounts of this protein were secreted from cells, indicating that the TM of G_N may also play a role in Golgi retention. It is also evident that the ectodomains of G_N and G_C interact with each other and that targeting of G_C to the Golgi apparatus is dependent upon its association with G_N , with ectodomain interactions being important.

In summary, although the CCHFV glycoproteins are unique in many aspects with respect to the glycoproteins from other members of the *Bunyaviridae* family, there are some similarities with regard to Golgi targeting and the glycoprotein subunits to which neutralizing antibodies are directed. Our studies indicate that CCHFV neutralization is likely to be context dependent and that more in-depth studies of various cell lines and animal models will be needed to characterize neutralization mechanisms and to identify antibodies that could be used therapeutically. Identification of regions on the CCHFV glycoproteins involved in viral neutralization, protection, and pro-

cessing will contribute to our understanding of the tropism and pathogenesis of this emerging viral pathogen.

ACKNOWLEDGMENTS

We thank Nicolette Pesik, Randall Bethke, and Loreen Hodgson for superb technical assistance on the production and characterization of the MABs. We also thank Aura Garrison and Donald Pijak for expert technical assistance.

This work was supported in part by National Institutes of Health training grant NIH T32 AI055400 and grant PR033269 from the Department of Defense Peer Reviewed Medical Research Program (PRMRP) of the Office of the Congressionally Directed Medical Research Programs.

REFERENCES

- Alexander, W. A., B. Moss, and T. R. Fuerst. 1992. Regulated expression of foreign genes in vaccinia virus under the control of bacteriophage T7 RNA polymerase and the *Escherichia coli lac* repressor. *J. Virol.* 66:2934-2942.
- Anderson, G. W., Jr., and J. F. Smith. 1987. Immunoelectron microscopy of Rift Valley fever viral morphogenesis in primary rat hepatocytes. *Virology* 161:91-100.
- Andersson, A. M., L. Melin, A. Bean, and R. F. Pettersson. 1997. A retention signal necessary and sufficient for Golgi localization maps to the cytoplasmic tail of a *Bunyaviridae* (Uukuniemi virus) membrane glycoprotein. *J. Virol.* 71:4717-4727.
- Andersson, A. M., L. Melin, R. Persson, E. Raschperger, L. Wikström, and R. F. Pettersson. 1997. Processing and membrane topology of the spike proteins G1 and G2 of Uukuniemi virus. *J. Virol.* 71:218-225.
- Andersson, A. M., and R. F. Pettersson. 1998. Targeting of a short peptide derived from the cytoplasmic tail of the G1 membrane glycoprotein of Uukuniemi virus (*Bunyaviridae*) to the Golgi complex. *J. Virol.* 72:9585-9596.
- Archer, D. B., D. J. Jeenes, D. A. MacKenzie, G. Brightwell, N. Lambert, G. Lowe, S. E. Radford, and C. M. Dobson. 1990. Hen egg white lysozyme expressed in, and secreted from, *Aspergillus niger* is correctly processed and folded. *Biotechnology (New York)* 8:741-745.
- Arikawa, J., I. Takashima, and N. Hashimoto. 1985. Cell fusion by haemorrhagic fever with renal syndrome (HFRS) viruses and its application for titration of virus infectivity and neutralizing antibody. *Arch. Virol.* 86:303-313.
- Arikawa, J., J. S. Yao, K. Yoshimatsu, I. Takashima, and N. Hashimoto. 1992. Protective role of antigenic sites on the envelope protein of Hantaan virus defined by monoclonal antibodies. *Arch. Virol.* 126:271-281.
- Aulitzky, W. E., T. F. Schulz, H. Tilg, D. Niederwieser, K. Larcher, L. Ostberg, M. Scriba, J. Martindale, A. C. Stern, P. Grass, et al. 1991. Human monoclonal antibodies neutralizing cytomegalovirus (CMV) for prophylaxis of CMV disease: report of a phase I trial in bone marrow transplant recipients. *J. Infect. Dis.* 163:1344-1347.
- Aulitzky, W. E., H. Tilg, D. Niederwieser, M. Hackl, B. Meister, and C. Huber. 1988. Ganciclovir and hyperimmunoglobulin for treating cytomegalovirus infection in bone marrow transplant recipients. *J. Infect. Dis.* 158:488-489.
- Bupp, K., K. Stillmock, and F. Gonzalez-Scarano. 1996. Analysis of the intracellular transport properties of recombinant La Crosse virus glycoproteins. *Virology* 220:485-490.
- Chen, S.-Y., and R. W. Compans. 1991. Oligomerization, transport, and Golgi retention of Punta Toro virus glycoproteins. *J. Virol.* 65:5902-5909.
- Chen, S.-Y., Y. Matsuoka, and R. W. Compans. 1991. Assembly and polarized release of Punta Toro virus and effects of brefeldin A. *J. Virol.* 65:1427-1439.
- Chen, S. Y., Y. Matsuoka, and R. W. Compans. 1991. Golgi complex localization of the Punta Toro virus G2 protein requires its association with the G1 protein. *Virology* 183:351-365.
- Chumakov, M. P., A. M. Butenko, N. V. Shalunova, L. I. Mart'ianova, S. E. Smirnova, N. Bashkirtsev, T. I. Zavodova, S. G. Rubin, E. A. Tkachenko, V. Karmysheva, V. N. Reingold, G. V. Popov, and A. P. Savinov. 1968. New data on the viral agent of Crimean hemorrhagic fever. *Vopr. Virusol.* 13:377. (In Russian.)
- Chumakov, M. P., S. E. Smirnova, and E. A. Tkachenko. 1970. Relationship between strains of Crimean hemorrhagic fever and Congo viruses. *Acta Virol.* 14:82-85.
- Englund, J. A., P. A. Piedra, and E. Whimbey. 1997. Prevention and treatment of respiratory syncytial virus and parainfluenza viruses in immunocompromised patients. *Am. J. Med.* 102:61-70, 75-76.
- Gerrard, S. R., and S. T. Nichol. 2002. Characterization of the Golgi retention motif of Rift Valley fever virus G_N glycoprotein. *J. Virol.* 76:12200-12210.
- Gonzalez-Scarano, F. 1985. La Crosse virus G1 glycoprotein undergoes a conformational change at the pH of fusion. *Virology* 140:209-216.

20. Gonzalez-Scarano, F., R. S. Janssen, J. A. Najjar, N. Pobjecky, and N. Nathanson. 1985. An avirulent G1 glycoprotein variant of La Crosse bunyavirus with defective fusion function. *J. Virol.* 54:757-763.
21. Grady, L. J., M. L. Sanders, and W. P. Campbell. 1983. Evidence for three separate antigenic sites on the G1 protein of La Crosse virus. *Virology* 126:395-397.
22. Grady, L. J., S. Srihongse, M. A. Grayson, and R. Deibel. 1983. Monoclonal antibodies against La Crosse virus. *J. Gen. Virol.* 64:1699-1704.
23. Hoogstraal, H. 1979. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. *J. Med. Entomol.* 15:307-417.
24. Huggins, J. W. 1989. Prospects for treatment of viral hemorrhagic fevers with ribavirin, a broad-spectrum antiviral drug. *Rev. Infect. Dis.* 11 (Suppl. 4): S750-S761.
25. Kingsford, L. 1984. Enhanced neutralization of La Crosse virus by the binding of specific pairs of monoclonal antibodies to the G1 glycoprotein. *Virology* 136:265-273.
26. Kingsford, L., K. H. Boucquey, and T. P. Cardoso. 1991. Effects of specific monoclonal antibodies on La Crosse virus neutralization: aggregation, inactivation by Fab fragments, and inhibition of attachment to baby hamster kidney cells. *Virology* 180:591-601.
27. Kingsford, L., L. D. Ishizawa, and D. W. Hill. 1983. Biological activities of monoclonal antibodies reactive with antigenic sites mapped on the G1 glycoprotein of La Crosse virus. *Virology* 129:443-455.
28. Kohler, G., S. C. Howe, and C. Milstein. 1976. Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines. *Eur. J. Immunol.* 6:292-295.
29. Kuusimäen, E., B. Bång, M. Hurme, and R. F. Pettersson. 1984. Uukuniemi virus maturation: immunofluorescence microscopy with monoclonal glycoprotein-specific antibodies. *J. Virol.* 51:137-146.
30. Ludwig, G. V., B. A. Israel, B. M. Christensen, T. M. Yuill, and K. T. Schultz. 1991. Monoclonal antibodies directed against the envelope glycoproteins of La Crosse virus. *Microb. Pathog.* 11:411-421.
31. Ludwig, G. V., B. A. Israel, B. M. Christensen, T. M. Yuill, and K. T. Schultz. 1991. Role of La Crosse virus glycoproteins in attachment of virus to host cells. *Virology* 181:564-571.
32. Malley, R., J. DeVincenzo, O. Ramilo, P. H. Dennehy, H. C. Meissner, W. C. Gruber, P. J. Sanchez, H. Jafri, J. Balsley, D. Carlin, S. Buckingham, L. Vernacchio, and D. M. Ambrosino. 1998. Reduction of respiratory syncytial virus (RSV) in tracheal aspirates in intubated infants by use of humanized monoclonal antibody to RSV F protein. *J. Infect. Dis.* 178:1555-1561.
33. Matsuoka, Y., S. Y. Chen, and R. W. Compans. 1991. Bunyavirus protein transport and assembly. *Curr. Top. Microbiol. Immunol.* 169:161-179.
34. Matsuoka, Y., S. Y. Chen, and R. W. Compans. 1994. A signal for Golgi retention in the bunyavirus G1 glycoprotein. *J. Biol. Chem.* 269:22565-22573.
35. Matsuoka, Y., S. Y. Chen, C. E. Holland, and R. W. Compans. 1996. Molecular determinants of Golgi retention in the Punta Toro virus G1 protein. *Arch. Biochem. Biophys.* 336:184-189.
36. Matsuoka, Y., T. Ihara, D. H. Bishop, and R. W. Compans. 1988. Intracellular accumulation of Punta Toro virus glycoproteins expressed from cloned cDNA. *Virology* 167:251-260.
37. Melin, L., R. Persson, A. Andersson, A. Bergström, R. Rönholm, and R. F. Pettersson. 1995. The membrane glycoprotein G1 of Uukuniemi virus contains a signal for localization to the Golgi complex. *Virus Res.* 36:49-66.
38. Morais, V. A., A. S. Crystal, D. S. Pijak, D. Carlin, J. Costa, V. M. Lee, and R. W. Doms. 2003. The transmembrane domain region of nicastrin mediates direct interactions with APh-1 and the gamma-secretase complex. *J. Biol. Chem.* 278:43284-43291.
39. Nabel, G. J. 1999. Surviving Ebola virus infection. *Nat. Med.* 5:373-374.
40. Nakitare, G. W., and R. M. Elliott. 1993. Expression of the Bunyamwera virus M genome segment and intracellular localization of NSm. *Virology* 195:511-520.
41. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193-199.
42. Olaleye, O. D., O. Tomori, and H. Schmitz. 1996. Rift Valley fever in Nigeria: infections in domestic animals. *Rev. Sci. Tech. Off. Int. Epizoot.* 15:937-946.
43. Onishchenko, G. G., V. I. Efremenko, N. G. Kovalev, N. A. Shibkov, M. Fedorov, N. Zhilina, G. N. Fedosova, G. V. Sysoliatina, M. Evchenko, A. P. Beier, V. A. Popov, K. V. Shenets, L. N. Marchukova, E. N. Afanas'ev, and N. V. Vasilenko. 2001. Specific epidemiologic features of Crimean hemorrhagic fever in Stavropol' region in 1999-2000. *Zh. Mikrobiol. Epidemiol. Immunobiol. Nov., Dec. (Suppl. 6):*86-89. (In Russian.)
44. Onishchenko, G. G., M. Lomov, V. I. Markov, V. A. Merkulov, A. N. Mironov, I. V. Borisevich, A. A. Makhlay, N. T. Vasil'ev, S. O. Vodop'ianov, N. G. Tikhonov, T. P. Pashanina, V. V. Manankov, V. P. Smelianski, S. T. Savchenko, and S. E. Smirnova. 2000. The laboratory diagnosis of an outbreak of hemorrhagic fever at Oblivskaya village, Rostov Province: proof of the etiological role of the Crimean-Congo hemorrhagic fever virus. *Zh. Mikrobiol. Epidemiol. Immunobiol. Mar., Apr.* 32-36. (In Russian.)
45. Onishchenko, G. G., V. I. Markov, V. A. Merkulov, N. T. Vasil'ev, A. M. Bereznoi, I. A. Androschuk, and V. A. Maksimov. 2001. Isolation and identification of Crimean-Congo hemorrhagic fever virus in the Stavropol territory. *Zh. Mikrobiol. Epidemiol. Immunobiol. Nov., Dec.* 7-11. (In Russian.)
46. Pekosz, A., C. Griot, K. Stillmock, N. Nathanson, and F. Gonzalez-Scarano. 1995. Protection from La Crosse virus encephalitis with recombinant glycoproteins: role of neutralizing anti-G1 antibodies. *J. Virol.* 69:3475-3481.
47. Pensiero, M. N., and J. Hay. 1992. The Hantaan virus M-segment glycoproteins G1 and G2 can be expressed independently. *J. Virol.* 66:1907-1914.
48. Pettersson, R. F., A. Andersson, and L. Melin. 1995. Mapping a retention signal for Golgi localization of a viral spike protein complex. *Cold Spring Harbor Symp. Quant. Biol.* 60:147-155.
49. Ruusala, A., R. Persson, C. S. Schmaljohn, and R. F. Pettersson. 1992. Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. *Virology* 186:53-64.
50. Saluzzo, J.-F., and B. Le Guenno. 1987. Rapid diagnosis of human Crimean-Congo hemorrhagic fever and detection of the virus in naturally infected ticks. *J. Clin. Microbiol.* 25:922-924.
51. Sanchez, A. J., M. J. Vincent, and S. T. Nichol. 2002. Characterization of the glycoproteins of Crimean-Congo hemorrhagic fever virus. *J. Virol.* 76:7263-7275.
52. Schmaljohn, C. S. 1996. *Bunyaviridae: the viruses and their replication*, p. 1447-1471. In B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Virology*, 3rd ed., vol. 1. Lippincott-Raven, Philadelphia, Pa.
53. Schmaljohn, C. S., J. Arikawa, S. E. Hasty, L. Rasmussen, H. W. Lee, P. W. Lee, and J. M. Dalrymple. 1988. Conservation of antigenic properties and sequences encoding the envelope proteins of prototype Hantaan virus and two virus isolates from Korean haemorrhagic fever patients. *J. Gen. Virol.* 69:1949-1955.
54. Schmaljohn, C. S., Y.-K. Chu, A. L. Schmaljohn, and J. M. Dalrymple. 1990. Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants. *J. Virol.* 64:3162-3170.
55. Schmaljohn, C. S., S. E. Hasty, and J. M. Dalrymple. 1992. Preparation of candidate vaccinia-vectored vaccines for haemorrhagic fever with renal syndrome. *Vaccine* 10:10-13.
56. Simmons, G., R. J. Wool-Lewis, F. Baribaud, R. C. Netter, and P. Bates. 2002. Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence. *J. Virol.* 76:2518-2528.
57. Trimble, R. B., and F. Maley. 1984. Optimizing hydrolysis of N-linked high-mannose oligosaccharides by endo-beta-N-acetylglucosaminidase H. *Anal. Biochem.* 141:515-522.
58. Vassilenko, S. M., T. L. Vassilev, L. G. Bozadjiev, I. L. Bineva, and G. Z. Kazarov. 1990. Specific intravenous immunoglobulin for Crimean-Congo haemorrhagic fever. *Lancet* 335:791-792.
59. Vincent, M. J., A. J. Sanchez, B. R. Erickson, A. Basak, M. Chretien, N. G. Seidah, and S. T. Nichol. 2003. Crimean-Congo hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1. *J. Virol.* 77:8640-8649.
60. Williams, R. J., S. Al-Busaidy, F. R. Mehta, G. O. Maupin, K. D. Wagoner, S. Al-Awaidey, A. J. Suleiman, A. S. Khan, C. J. Peters, and T. G. Ksiazek. 2000. Crimean-Congo haemorrhagic fever: a seroepidemiological and tick survey in the Sultanate of Oman. *Trop. Med. Int. Health* 5:99-106.
61. Wood, O. L., V. H. Lee, J. S. Ash, and J. Casals. 1978. Crimean-Congo hemorrhagic fever, Thogoto, Dugbe, and Jos viruses isolated from ixodid ticks in Ethiopia. *Am. J. Trop. Med. Hyg.* 27:600-604.
62. Zhang, X. K., I. Takashima, and N. Hashimoto. 1989. Characteristics of passive immunity against hantavirus infection in rats. *Arch. Virol.* 105:235-246.

Presence of broadly reactive and group-specific neutralizing epitopes on newly described isolates of *Crimean-Congo hemorrhagic fever virus*

Asim A. Ahmed,¹ Jeanne M. McFalls,¹ Christian Hoffmann,¹
Claire Marie Filone,¹ Shaun M. Stewart,¹ Jason Paragas,²
Shabot Khodjaev,³ Dilbar Shermukhamedova,³ Connie S. Schmaljohn,²
Robert W. Doms¹ and Andrea Bertolotti-Ciarlet¹

Correspondence

Andrea Bertolotti-Ciarlet
aciarlet@mail.med.upenn.edu

¹Department of Microbiology, University of Pennsylvania, Philadelphia, PA 19104, USA

²Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702, USA

³Institute of Virology, Ministry of Health, Tashkent, Uzbekistan

Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus *Nairovirus* of the family *Bunyaviridae*, causes severe disease in humans with high rates of mortality. The virus has a tripartite genome comprising a small (S), a medium (M) and a large (L) RNA segment; the M segment encodes the two viral glycoproteins, G_N and G_C. Whilst relatively few full-length M segment sequences are available, it is apparent that both G_N and G_C may exhibit significant sequence diversity. It is unknown whether considerable antigenic differences exist between divergent CCHFV strains, or whether there are conserved neutralizing epitopes. The M segments derived from viral isolates of a human case of CCHF in South Africa (SPU 41/84), an infected tick (*Hyalomma marginatum*) in South Africa (SPU 128/81), a human case in Congo (UG 3010), an infected individual in Uzbekistan (U2-2-002) and an infected tick (*Hyalomma asiaticum*) in China (Hy13) were sequenced fully, and the glycoproteins were expressed. These novel sequences showed high variability in the N-terminal region of G_N and more modest differences in the remainder of G_N and in G_C. Phylogenetic analyses placed these newly identified strains in three of the four previously described M segment groups. Studies with a panel of mAbs specific to G_N and G_C indicated that there were significant antigenic differences between the M segment groups, although several neutralizing epitopes in both G_N and G_C were conserved among all strains examined. Thus, the genetic diversity exhibited by CCHFV strains results in significant antigenic differences that will need to be taken into consideration for vaccine development.

Received 10 May 2005

Accepted 22 August 2005

INTRODUCTION

Crimean-Congo hemorrhagic fever virus (CCHFV) causes a haemorrhagic disease in humans with mortality rates that range from 10 to 80 % (Whitehouse, 2004). CCHFV can be isolated from ticks, livestock and humans (Whitehouse, 2004). Infection can occur either through the bite of an infected tick, exposure to tissue and fluids from an infected animal or through contact with infected human bodily fluids. CCHFV infection was first described during an outbreak in Russia during the 1940s, when more than 200 cases of severe haemorrhagic fever were reported among agricultural workers and soldiers in the Crimean peninsula

(Chumakov *et al.*, 1968, 1970). Since then, the virus has spread or has been recognized throughout many regions of the world, including sub-Saharan Africa (Williams *et al.*, 2000; Wood *et al.*, 1978), Bulgaria, the Arabian Peninsula, Iraq, Pakistan, the former Yugoslavia, northern Greece and north-west China (Chumakov *et al.*, 1970; Hoogstraal, 1979; Olaleye *et al.*, 1996; Onishchenko *et al.*, 2000, 2001a, b).

CCHFV is a member of the genus *Nairovirus* within the family *Bunyaviridae* (Schmaljohn, 1996). Members of this enveloped virus family have a tripartite, single-stranded RNA genome of negative polarity. The small segment (S) encodes the viral nucleocapsid, the medium segment (M) encodes the two glycoproteins, G_N and G_C, and the large segment (L) encodes an RNA-dependent RNA polymerase. The viral glycoproteins, like those of other members of the family *Bunyaviridae*, are synthesized as a polyprotein

The GenBank/EMBL/DBJ accession numbers for the sequences described in this paper are AY900141–AY900145.

Supplementary figures are available in JGV Online.

precursor (Schmaljohn, 1996) that undergoes proteolytic cleavage events to yield the mature glycoproteins (Vincent *et al.*, 2003). The G_N precursor protein (Pre- G_N) contains an N-terminal domain with a high proportion of Ser, Thr and Pro residues. This region resembles the mucin-like domain present in the glycoproteins of other viruses, most notably the Ebola virus glycoprotein (Simmons *et al.*, 2002).

The G_N and G_C glycoproteins of CCHFV probably influence the host range, cell tropism and pathogenicity of this vertebrate and tick virus, and are the targets for neutralizing antibodies. Studies thus far indicate that portions of G_N are highly variable compared with other regions of G_N and with G_C (Chinikar *et al.*, 2004; Hewson *et al.*, 2004a, b; Morikawa *et al.*, 2002; Papa *et al.*, 2002). However, there is limited sequence information available on CCHFV isolates from regions outside China and the former Soviet Union (Chinikar *et al.*, 2004; Hewson *et al.*, 2004a, b; Morikawa *et al.*, 2002; Papa *et al.*, 2002). We previously described the first neutralizing mAbs to CCHFV (Bertolotti-Ciarlet *et al.*, 2005). In addition, some of these antibodies were shown to be protective in a suckling mouse animal model (Bertolotti-Ciarlet *et al.*, 2005). However, it is not clear whether significant antigenic differences exist between divergent CCHFV isolates or whether conserved neutralizing epitopes are present. This information is important for vaccine development, as the identification of conserved neutralizing epitopes may lead to the development of vaccines and entry inhibitors.

To further characterize the genetic diversity of the CCHFV M segment, we cloned and expressed glycoproteins from divergent CCHFV strains that were passaged a limited number of times. Additionally, to assess antigenic differences between CCHFV isolates, we cloned and fully sequenced the open reading frames from five CCHFV isolates obtained from humans or ticks in South Africa, Congo, Uzbekistan and China. Phylogenetic analyses indicated that one or more of these new strains segregated with three of the four previously described M segment groups (Hewson *et al.*, 2004b). The glycoproteins from each strain were expressed transiently in cell lines and their ability to be recognized by a panel of mAbs to G_N and G_C was determined. The genetic proximity of strains and their antigenic similarity were imperfectly correlated. Whilst some epitopes were conserved, others were not, indicating that CCHFV vaccines designed to induce neutralizing antibodies may have to include immunogens derived from several CCHFV strains, or in some way focus the immune response on conserved neutralizing epitopes.

METHODS

Virus strains and cells. African green monkey kidney fibroblast (CV-1), Vero, Vero E6, human cervix carcinoma (HeLa) and human embryonic kidney (HEK-293T) cells, obtained from the ATCC (Manassas, VA, USA), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Similarly, the human tumour cell line

SW-13 (adrenocortical carcinoma) was grown in DMEM supplemented with 2.5% FBS. CCHFV strains Hy13, U2-2-002, SPU 41/84, SPU 128/81, SPU 94/87 and UG 3010 were used in this study. All of the viruses were passaged by intracerebral inoculation of 1-day-old mice with each CCHFV isolate, using a dose resulting in the death of 50% of the mice. The mice were killed 24 h post-infection and the brain was harvested. Brains were homogenized to 10% (w/v) with Hanks' salt solution and clarified by centrifugation at 10 000 r.p.m. for 30 min. CCHFV prototype strain IbAr10200, first isolated in 1976 from ticks (*Hyalomma excavatum*) from Sokoto, Nigeria, was grown in African green monkey kidney Vero or Vero E6 cells (Sanchez *et al.*, 2002). Republic of South Africa CCHFV strain SPU 41/84 was isolated from an infected human in 1984 and passaged in suckling mice four times. Republic of South Africa CCHFV strain SPU 128/81 was isolated in 1981 from infected ticks (*Hyalomma marginatum rufipes*) and passaged in suckling mice three times. Congolese strain UG 3010 was isolated in 1956 from a physician that became ill after handling blood taken from an infected boy at the Kisangani Hospital. This was one of the first 'Congo' strains isolated (Simpson *et al.*, 1967; Woodall *et al.*, 1967). Chinese strain Hy13 was isolated from infected ticks (*H. marginatum rufipes*) in 1968 and was passaged in suckling mice three times. CCHFV strain U2-2-002/U-6415 from Uzbekistan was isolated from an infected human and passaged in suckling mice four times. All work with replication-competent CCHFV was conducted in a biosafety level 4 facility at the US Army Medical Research Institute of Infectious Diseases (USAMRIID).

RNA purification, RT-PCR and sequencing. Consensus primers were designed based on an alignment of known full-length M segment sequences available in GenBank. In order to amplify the 5' half of the M segment from each strain, primers CCHF 5' (5'-TCTCAAAGAAACACGTGCCGC-3') and CCHF 3519 R (5'-GTACTCRAAGACAGGRGARTACAT-3') were designed. CCHF 2325 F (5'-AATGCAATAGAYGCTGARATGCA-3') and CCHF 3'R (5'-TCTCAAAGAWATAGTGGCGGCACGCAGTC-3') were designed to amplify the 3' half of the M segment for each strain. Wobble code includes R=A or G, Y=C or T and W=A or T. The two designed amplicons share 1 kb overlapping sequence at the centre of the M segment. This strategy of amplification of the M segment in two halves was utilized for most of the strains. Total RNA was isolated from lysates of SW-13 cells infected with the different CCHFV strains by utilizing TRIzol LS (Invitrogen) and removed from bio-containment. The samples were chloroform-extracted, followed by high-speed centrifugation and isolation of the resulting aqueous layer. RNA was precipitated by using propan-2-ol and pellets were resuspended in RNase-free distilled water. RNA was further purified through the RNeasy system (Qiagen) according to the manufacturer's directions.

Reverse transcription of the entire M RNA segment was performed by using 5 µl RNA from above, using CCHF 3'R (300 ng) and 1 µl of a mixture of the four dNTPs (at 10 µM each) in 12 µl. This mixture was heated to 65 °C for 5 min and chilled rapidly on ice. Four microlitres of 5 × RT buffer, 2 µl 0.1 M dithiothreitol and RNasin (40 U) were added to the mixture and heated to 42 °C for 2 min. Then, 1 µl Superscript II (Invitrogen) reverse transcriptase (RT, 200 U) was added to the reaction mixture and incubated at 42 °C for 1 h. The resultant cDNA generated from this reaction was then used as a template in subsequent PCRs. PCR was performed by using 2 µl cDNA generated from the RT reaction, 5 µl CCHF primers (10 µM each), 5 µl 10 × PCR buffer, 1.5 µl dNTP mixture, 2 µl MgSO₄ (50 µM) and 0.6 µl Hi-Fidelity polymerase (5 U) in a 50 µl reaction. PCR thermocycler conditions were used as recommended by the manufacturer with an annealing temperature of 45 °C. When the consensus primer set was unable to generate a PCR product for one half of the M segment, a gene-specific internal primer was designed based on sequences from

the half of the M segment that did yield a product. This was the case with UG3010; the 3' half of the UG3010 M segment was amplified by using an gene-specific internal primer, 3370F (5'-TGAACACAGGGGCAACAAAATC-3'), in combination with the 3' external consensus primer CCHF 3'R. Resultant PCR products were TA-cloned into pCR4-TOPO using the TOPO cloning for sequencing system (Invitrogen) according to the manufacturer's instructions. Recombinant clones were confirmed by sequencing in both directions. On average, three clones from two PCRs were sequenced in both directions to generate a sequence for each M segment half. By using the data from the 5' and 3' ends of each M segment that shared a 1 kb overlap, the sequence of each strain's M segment was resolved. These two overlapping fragments were utilized for cloning a full-length M segment into the expression vector pCAGGS (Niwa *et al.*, 1991). The sequences have been deposited in GenBank (accession numbers AY900141–AY900145).

Mapping of mAb 11E7. In order to map the epitope recognized by mAb 11E7, we constructed expression plasmids that represent fragments of the G_C ectodomain. Primers were synthesized according to the published sequence for strain IbAr10200 (Sanchez *et al.*, 2002) and standard PCR technology was performed to clone the amplicons into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen). The 5' primers included the CACC sequence at the 5' end and the start codon to allow for directional cloning. The 3' primers did not possess a stop codon to allow the inclusion of the V5 cassette and polyhistidine epitope tags at the C terminus of the protein. Cloning was performed as described by the manufacturer (Invitrogen) and all constructs were sequenced. All primer sequences are available upon request.

Protein analysis. To analyse protein expression, HEK-293T cells were infected with recombinant vaccinia virus vTF1.1 expressing T7 polymerase (Alexander *et al.*, 1992) and transfected 40 min later by using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, cell extracts were prepared in 50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100 and Complete Protease Inhibitor cocktail (Roche Applied Sciences). Cell lysates were incubated at 4°C for 3 min and then centrifuged at 10 000 *g* for 10 min. The supernatant was mixed with sample buffer [0.08 M Tris/HCl (pH 6.8), 2% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.005% bromophenol blue] and incubated at 56°C for 10 min before electrophoresis in a Criterion SDS-PAGE 4–15% Tris/HCl gel (Bio-Rad). Western blot analysis was performed by using mouse anti-V5 (Invitrogen) or mAb 11E7 as primary antibodies and sheep anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) followed by visualization with ECL-Plus Western blotting detection reagents (Bioscience). In the case of Western blotting developed with mAb 11E7, samples were not treated with β -mercaptoethanol.

Immunofluorescence (IF) microscopy. To determine whether there were antigenic differences among glycoproteins from different CCHFV strains and to characterize their localization within cells, we performed indirect IF microscopy as described previously (Morais *et al.*, 2003). HeLa cells grown to 50% confluence on glass coverslips were transfected with the different pCAGGS plasmids containing the CCHFV M segments. At 24 h post-transfection, the cells were fixed with 2% (w/v) formaldehyde in PBS, permeabilized with 0.5% Triton X-100 and stained with ascites containing a G_N- or G_C-specific mAb, diluted 1:250 in PBS containing 0.5 mM MgCl₂ and 4% FBS. Then, cells were washed with PBS and incubated for 1 h with the secondary antibody conjugated to Alexa Fluor 488 (goat anti-mouse) (Molecular Probes) diluted 1:500 in PBS containing 4% FBS. Finally, cells were washed in PBS, mounted with Fluoromount-G (Southern Biotechnology Associates) and examined on a Nikon E600 microscope at $\times 60$ magnification utilizing UV illumination.

Sequence analysis. We studied the relationships between the newly sequenced CCHFV M segments and previously published

full-length isolates. The sequence alignments were produced by using CLUSTAL_X (Thompson *et al.*, 1997) and checked manually for accuracy. The phylogenetic trees were drawn by using the PHYLIP package version 3.57c (Felsenstein, 1997). Briefly, the trees were obtained by using distance methods; SEQBOOT was used to obtain 1000 bootstrap replications of the original sequence alignment. The bootstrapped alignments were used for construction of a consensus tree with NEIGHBOR and CONSENSE as described in the package documentation. Distance between species shown in Fig. 1 was obtained from the original alignment. Consensus trees were rooted with the Dugbe strain, using TREEVIEW version 1.6.1 (Page, 1996).

RESULTS AND DISCUSSION

Cloning and expression of M segments from diverse regions of the world

There is limited sequence information on CCHFV isolates from regions outside China and the former Soviet Union, with only one full-length M segment from an African strain being described (Chinikar *et al.*, 2004; Hewson *et al.*, 2004a, b; Morikawa *et al.*, 2002; Papa *et al.*, 2002). It is not known whether divergent CCHFV strains exhibit significant antigenic variability or share neutralizing epitopes – information that is important for vaccine development. In addition, only the glycoproteins of the extensively passaged IbAr10200 and Matin strains have been well characterized with regards to processing and cellular localization (Sanchez *et al.*, 2002). To define the genetic and antigenic diversity of geographically diverse CCHFV strains, we cloned, sequenced and expressed the M segments from five isolates. Congolese strain UG3010 was isolated in 1956 from a physician that became ill after handling blood taken from an infected boy at the Kisangani Hospital. This was one of the first 'Congo' strains isolated (Simpson *et al.*, 1967; Woodall *et al.*, 1967). Republic of South Africa CCHFV strain SPU 41/84 was isolated from a patient in South Africa in 1984 (Blackburn *et al.*, 1987), whilst Republic of South Africa strain SPU 128/81 was isolated from *H. marginatum* ticks (Shepherd *et al.*, 1985). Chinese strain Hy13 was isolated from *H. marginatum rufipes* ticks in Xinjiang, China, and Uzbekistan strain U2-2-002/U-6415 was isolated from *Alveonasus lahorensis* ticks. The viruses were passaged in suckling mice for between three and 11 times, as described in Methods.

M segment phylogeny

Hewson *et al.* (2004b) thoroughly described CCHFV phylogeny, revealing the existence of four M segment groups termed M1, M2, M3 and M4. We found that Chinese strain Hy13 clustered with group M1, along with several other Chinese strains and Pakistan strain Matin (Fig. 1a). South African strains SPU 41/84 and SPU 128/81 and Uzbekistan strain U2-2-002 clustered with group M2, along with previously described strains from China, Uzbekistan, Pakistan, Iraq and Nigeria (Hewson *et al.*, 2004b; Morikawa *et al.*, 2002; Sanchez *et al.*, 2002). Congo strain UG3010 clustered with group M3, which contains two previously described Chinese strains (Fig. 1a) (Morikawa *et al.*, 2002). As noted previously, whilst there is some geographical

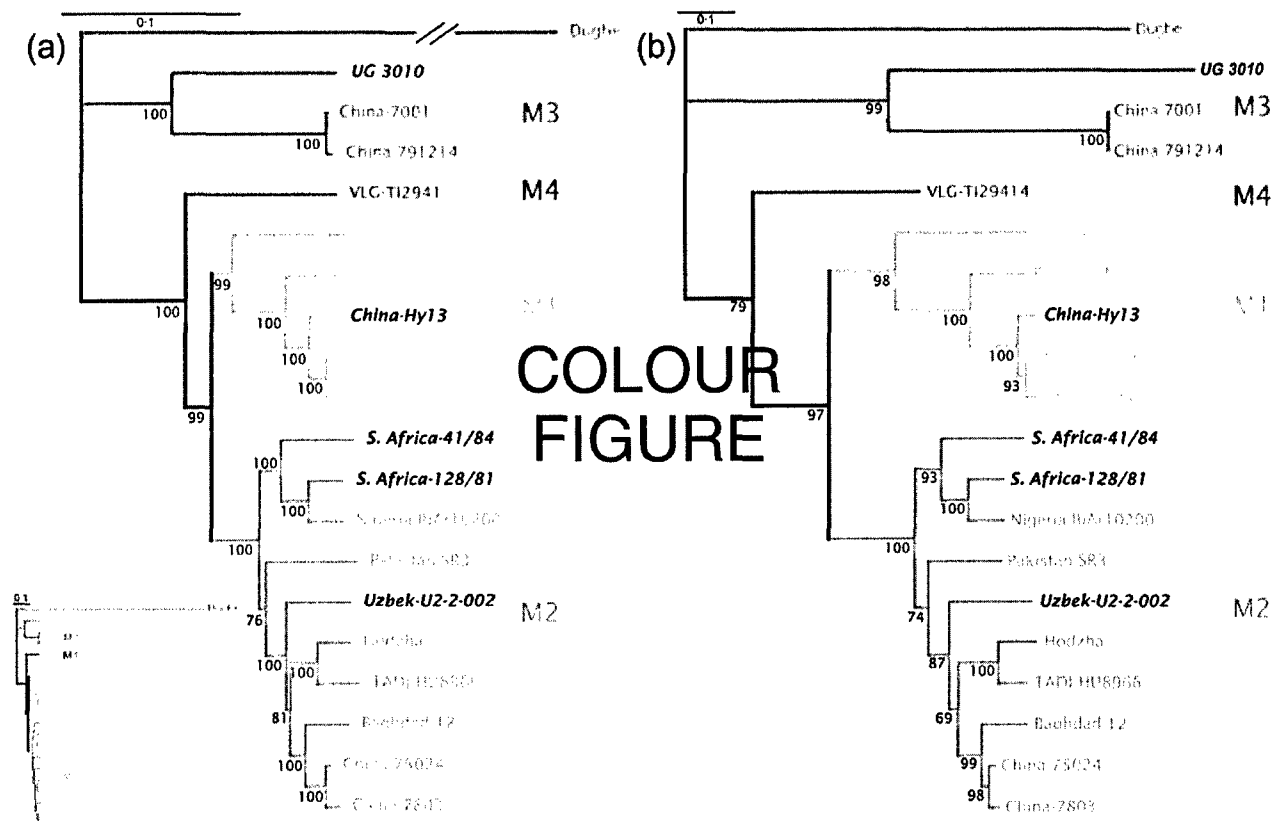


Fig. 1. Phylogenetic trees showing the relationships between CCHFV M segments. The bootstrap values shown are percentages of 1000 replications of the original dataset. All sequences were retrieved from GenBank. Strains marked in bold were sequenced as part of this study (GenBank accession numbers AY900141–AY900145). (a) Phylogenetic tree constructed by utilizing the full-length M segment sequence. The branch length for the Dugbe sequence (outgroup) was cropped for presentation purposes. The small tree at the bottom left of the figure shows the correct branch-length relationship between Dugbe and the remaining sequences. (b) Phylogenetic tree constructed with only the mucin-like domain sequence. Bars, 0.1 substitution per base position.

clustering of CCHFV strains, there are also examples of geographically distant but genetically closely related virus isolates, perhaps reflecting trade in livestock or dispersal of infected ticks by migratory birds (Hewson *et al.*, 2004b).

We repeated the phylogenetic analysis of the strains using different regions of the M segment (the mucin-like domain or P35 domains of G_N , G_N lacking these domains, and G_C). The same phylogenetic tree was obtained in all cases (data not shown), even when only the highly variable mucin-like domain was used (Fig. 1b). This indicates that sequencing only a small portion of the M segment should make it possible to categorize new CCHFV isolates accurately.

Pairwise analysis of M segments sequences

The five completed M segment sequences had lengths ranging from 1684 to 1699 aa. The CCHFV glycoprotein precursor has been described to contain 78–80 cysteine residues on average, suggesting the presence of an exceptionally large number of disulfide bonds and a complex

secondary structure. Cysteine residues were highly conserved, as were the sequences at the predicted proteolytic cleavage sites that have been described previously (Vincent *et al.*, 2003). The number of potential N-linked glycosylation sites ranged from nine to 14. The M segments of the newly described strains were compared with published sequences and identity values were calculated with the CLUSTAL_X program (Jeanmougin *et al.*, 1998; Thompson *et al.*, 1997), and a matrix was constructed by using the program BioEdit (Tippmann, 2004). The G_N precursor protein (Pre- G_N) contains a highly variable domain at its N terminus that contains a high proportion of serine, threonine and proline residues, and it is predicted to be heavily O-glycosylated, thus resembling a mucin-like domain present in other viral glycoproteins, most notably the Ebola virus glycoprotein. This analysis confirmed that the mucin-like domain is highly variable (Table 1) (Hewson *et al.*, 2004a, b; Morikawa *et al.*, 2002; Sanchez *et al.*, 2002). When the identity values for the M segments were calculated based only on the mucin domain, the M1, M2, M3 and M4 strains were clearly distinct, consistent with the phylogenetic analyses (Table 1).

Table 1. Complete M segment deduced amino acid identities of CCHFV virus strains
ID, Identical. Shading indicates relative levels of similarity (white, > 0.8; dark, < 0.2).

Mucin sequence																				
Strain		M3			M4	M1					M2									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. Dugbe	ID	0.08	0.09	0.09	0.11	0.10	0.11	0.12	0.12	0.11	0.10	0.12	0.10	0.11	0.11	0.09	0.09	0.09	0.10	0.10
2. UG 3010	0.08	ID										0.17	0.18	0.20		0.19	0.19	0.20	0.19	
3. China-7001	0.09		ID	1.00																
4. China-791214	0.09		1.00	ID																
5. VLG-TI29414	0.11				ID															
6. China-BA66019	0.10					ID	0.81													
7. Pakistan-Matin	0.11					0.81	ID	0.78	0.77	0.76										
8. China-Hy13	0.12						0.78	ID	0.94	0.92										
9. China-BA88166	0.12						0.77	0.94	ID	0.98										
10. China-BA8402	0.11						0.76	0.92	0.98	ID										
11. S. Africa 128/81	0.10	0.17									ID	0.72	0.72	0.65	0.60	0.62	0.62	0.63	0.65	0.64
12. S. Africa 41/84	0.12	0.18									0.72	ID	0.86	0.71	0.66	0.67	0.68	0.65	0.72	0.71
13. Nigeria-IbAr10200	0.10	0.20									0.72	0.86	ID	0.69	0.66	0.65	0.68	0.65	0.71	0.71
14. Pakistan-SR3	0.11										0.65	0.71	0.69	ID	0.69	0.72	0.73	0.72	0.75	0.74
15. Uzbek-U2-2-002	0.11	0.19									0.65	0.66	0.65	0.69	ID	0.69	0.72	0.73	0.77	0.76
16. Hodzha	0.09	0.19									0.62	0.67	0.65	0.72	0.69	ID	0.87	0.73	0.78	0.78
17. TADJ-HU8966	0.09	0.20									0.62	0.66	0.66	0.73	0.72	0.87	ID	0.74	0.81	0.81
18. Baghdad-12	0.09	0.19									0.63	0.65	0.65	0.72	0.73	0.73	0.74	ID	0.89	0.88
19. China-75024	0.10										0.65	0.72	0.71	0.75	0.77	0.78	0.81	0.89	ID	0.97
20. China-78033	0.10										0.64	0.71	0.71	0.74	0.76	0.76	0.81	0.88	0.87	ID
Full-length sequence																				
Strain		M3			M4	M1					M2									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. Dugbe	ID																			
2. UG 3010		ID	0.82	0.82	0.72	0.72	0.73	0.73	0.73	0.73	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72	0.72
3. China-7001		0.82	ID	1.00	0.73	0.72	0.74	0.74	0.74	0.73	0.73	0.73	0.73	0.73	0.73	0.73	0.72	0.73	0.73	0.72
4. China-791214		0.82	1.00	ID	0.73	0.72	0.73	0.74	0.73	0.73	0.72	0.72	0.73	0.73	0.72	0.73	0.72	0.73	0.73	0.72
5. VLG-TI29414		0.72	0.73	0.73	ID	0.82	0.82	0.83	0.83	0.83	0.82	0.83	0.83	0.82	0.83	0.83	0.82	0.83	0.83	0.83
6. China-BA66019		0.72	0.72	0.72	0.82	ID	0.88	0.88	0.88	0.88	0.86	0.84	0.85	0.85	0.85	0.85	0.84	0.85	0.85	0.85
7. Pakistan-Matin		0.73	0.74	0.73	0.82	0.88	ID	0.94	0.94	0.94	0.86	0.85	0.86	0.85	0.85	0.86	0.85	0.86	0.86	0.86
8. China-Hy13		0.73	0.74	0.74	0.83	0.88	0.94	ID	0.97	0.97	0.85	0.85	0.85	0.85	0.85	0.86	0.85	0.86	0.86	0.85
9. China-BA88166		0.73	0.74	0.73	0.83	0.88	0.94	0.97	ID	0.99	0.85	0.85	0.85	0.85	0.86	0.86	0.85	0.86	0.86	0.86
10. China-BA8402		0.73	0.73	0.73	0.83	0.88	0.94	0.97	0.99	ID	0.85	0.85	0.85	0.85	0.85	0.86	0.85	0.85	0.86	0.85
11. S. Africa 128/81		0.72	0.73	0.72	0.82	0.86	0.86	0.85	0.85	0.85	ID	0.92	0.96	0.90	0.90	0.91	0.90	0.90	0.92	0.91
12. S. Africa 41/84		0.72	0.73	0.72	0.83	0.84	0.85	0.85	0.85	0.85	0.92	ID	0.92	0.89	0.89	0.90	0.89	0.90	0.90	0.90
13. Nigeria-IbAr10200		0.72	0.73	0.73	0.83	0.85	0.86	0.85	0.85	0.85	0.96	0.92	ID	0.90	0.90	0.90	0.90	0.91	0.91	0.91
14. Pakistan-SR3		0.72	0.73	0.73	0.82	0.85	0.85	0.85	0.85	0.85	0.90	0.89	0.90	ID	0.90	0.90	0.90	0.90	0.91	0.91
15. Uzbek-U2-2-002		0.72	0.73	0.72	0.83	0.85	0.85	0.85	0.86	0.85	0.90	0.89	0.90	0.90	ID	0.93	0.92	0.93	0.94	0.94
16. Hodzha		0.72	0.73	0.73	0.83	0.85	0.86	0.86	0.86	0.86	0.91	0.90	0.90	0.90	0.93	ID	0.96	0.93	0.95	0.94
17. TADJ-HU8966		0.72	0.72	0.72	0.82	0.84	0.85	0.85	0.85	0.85	0.90	0.89	0.90	0.90	0.92	0.96	ID	0.92	0.94	0.94
18. Baghdad-12		0.72	0.73	0.73	0.83	0.85	0.86	0.86	0.86	0.85	0.90	0.90	0.90	0.90	0.93	0.93	0.92	ID	0.96	0.96
19. China-75024		0.72	0.73	0.73	0.83	0.85	0.86	0.86	0.86	0.86	0.92	0.90	0.91	0.91	0.94	0.95	0.94	0.96	ID	0.99
20. China-78033		0.72	0.72	0.72	0.83	0.85	0.86	0.85	0.86	0.85	0.91	0.90	0.91	0.91	0.94	0.94	0.94	0.96	0.99	ID

When the same type of comparison was performed by using the full-length sequences or other portions of G_N or G_C , distinctions between the subgroups were not as obvious (data not shown), although the M3 group was the best-defined and -differentiated of the four subgroups (Table 1).

Antigenic analysis of G_N and G_C

Antigenic variation of arboviruses is of relevance because it may provide clues on the possible directions of epidemics or endemic spread. Little is known about antigenic relationships among CCHFV strains, in part because of the lack of adequate reagents. Early studies on antigenic relationships between CCHFV strains have shown that strains from diverse parts of the world have close antigenic relationships (Tignor *et al.*, 1980). However, these studies were performed by utilizing polyclonal serum obtained from animals inoculated with infected mouse-brain tissue, which normally results mainly in antibodies directed against the nucleocapsid (Blackburn *et al.*, 1987). Indeed, with the exception of a recent report from our laboratory (Bertolotti-Ciarlet *et al.*, 2005), the CCHFV mAbs described thus far are directed against the nucleocapsid (Blackburn *et al.*, 1987). The viral glycoproteins might exhibit a degree of higher antigenic variability than the nucleocapsid protein as a result of immune selection and the adaptation needed to efficiently bind to and enter diverse cell types. Therefore, we determined antigenic differences between G_N and G_C from

different strains, utilizing a panel of eight mAbs to G_N and nine mAbs to G_C (Bertolotti-Ciarlet *et al.*, 2005). These mAbs bind to conformation-dependent epitopes and so were characterized for their ability to recognize the different G_N and G_C proteins by IF microscopy utilizing constructs expressing only one of the glycoproteins (Bertolotti-Ciarlet *et al.*, 2005; Morais *et al.*, 2003). The G_N and G_C proteins from each of the five strains were recognized by a subset of the mAbs and were localized to both the endoplasmic reticulum and the Golgi, consistent with correct processing and transport (Table 2 and Fig. 2) (Andersson & Pettersson, 1998; Andersson *et al.*, 1997a, b; Chen & Compans, 1991; Chen *et al.*, 1991; Gerrard & Nichol, 2002). The Golgi localization was confirmed by IF microscopy using a marker for TGN46 (Serotec), a sheep antibody specific for a heavily glycosylated protein localized primarily in the trans-Golgi network (data not shown). The M segments from each of the five virus strains appeared to be expressed at similar levels, as they were all recognized well by mAb 11E7 (see Supplementary Fig. S1, available in JGV Online). In addition, by using a rabbit polyclonal serum, we were able to show that the G_N glycoproteins from each of the five CCHFV strains were expressed and processed properly (see Supplementary Fig. S2, available in JGV Online). With regards to mAb reactivity, two of the M2 group strains (SPU 128/81 and U2-2-002) were virtually identical to IbAr10200, which itself is an M2 group strain. However, the closely related SPU 41/84 M2 strain was not recognized by two of

Table 2. Reactivity of IbAr10200 mAbs with different CCHFV strains

The characterization of neutralization and protection has been described previously (Bertolotti-Ciarlet *et al.*, 2005). Neutralization is shown as the plaque-reduction neutralization titre (PRNT 80%) and protection data as the number of surviving mice compared with the total number of mice treated. +, Positive signal by IF; -, negative result. The identity of the antibodies was determined by IF analysis using constructs that contain G_N or G_C alone.

Target	mAb	Neutralization	Protection (%)	Hy13 M1	IbAr10200 M2	U2-2-002 M2	SPU 128/81 M2	SPU 41/84 M2	UG 3010 M3
G_C	11E7	2560	70	+	+	+	+	+	+
	3E3	<20	45	+	+	+	+	+	+
	12A9	>5120	75	-	+	+	+	+	-
	8A1	>5120	100	-	+	+	+	+	+
	30F7	2560	40	-	+	+	+	+	+
	7C12	<40	40	-	+	+	+	+	+
	9H3	>5120	50	-	+	+	+	+	+
	5B5	1280	50	-	+	+	+	-	+
	4C1	>5120	41	+	+	+	+	-	+
G_N	5A5	<40	60	+	+	+	+	+	+
	9C6	<40	20	+	+	+	+	+	+
	8F10	<40	70	+	+	+	+	+	+
	11F6	<40	30	-	+	-	+	+	+
	7F5	<40	10	+	+	+	+	-	+
	2C9	<40	30	+	+	+	+	-	-
	3F3	<40	60	+	+	+	+	+	-
	10G4	<40	75	+	+	+	+	+	-

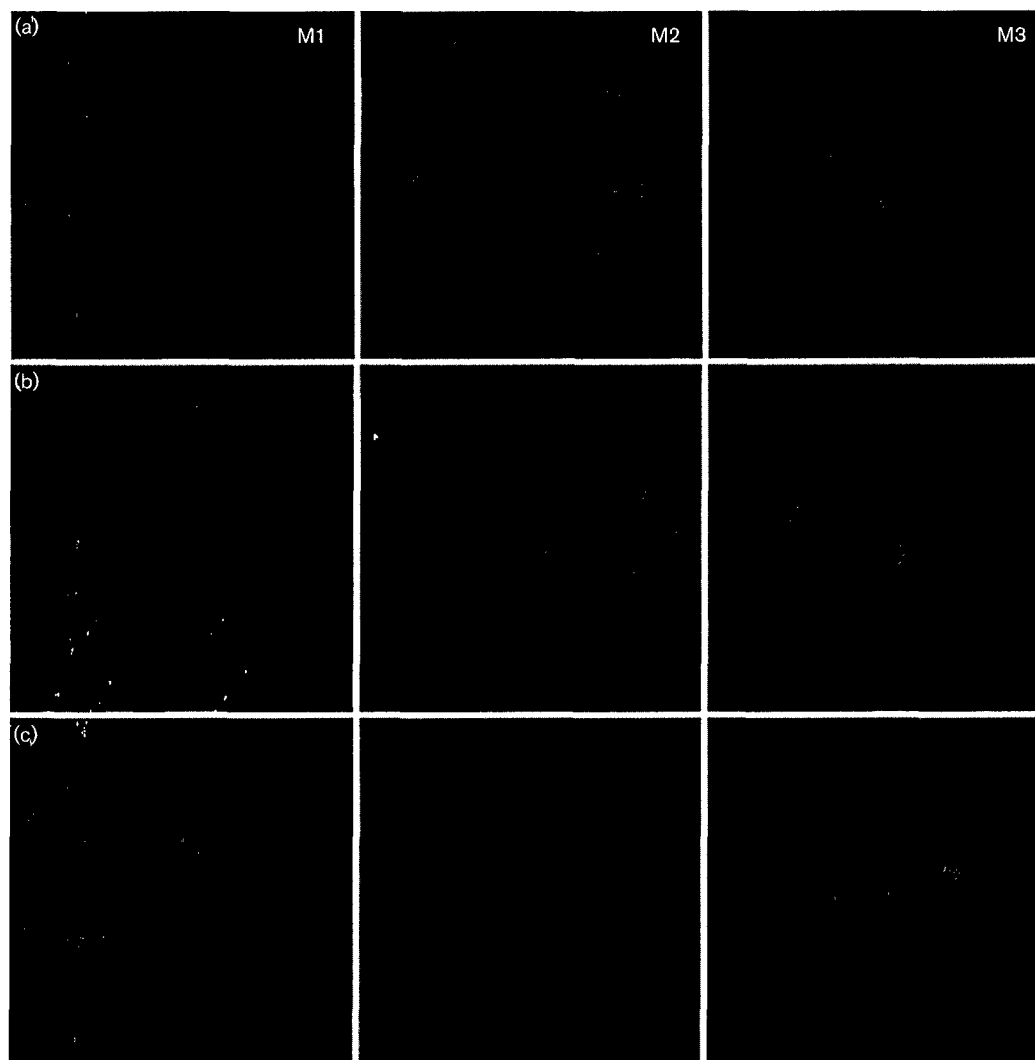


Fig. 2. IF analysis of CCHFV G_N and G_C glycoproteins. Transfected HeLa cells expressing CCHFV glycoproteins from different virus strains were processed for IF microscopy and stained with each of 17 different mouse anti-CCHFV mAbs (red) as described previously (Bertolotti-Ciarlet *et al.*, 2005). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Representative examples are shown in the following panels: (a) 11E7 anti- G_C mAb, from left to right: CCHFV strains M1 Hy13, M2 SPU 128/81, M3 UG 3010; (b) 8A1 anti- G_C mAb, from left to right: M1 Hy13, M2 U2-2-002, M3 UG 3010; (c) 7F5 anti- G_N mAb, from left to right: M1 Hy13, M2 SPU 41/84, M3 UG 3010. HeLa cells were transfected with CCHFV M segments by using Lipofectamine 2000 (Invitrogen) and processed 24 h later.

the G_C mAbs or by two of the G_N mAbs (Table 2). On the other hand, the M1 group strain Hy13 was recognized by seven of the eight G_N mAbs, but by only three of nine G_C mAbs. The M3 strain UG 3010, which was genetically the most distantly related to IbAr10200, shared a high degree of antigenic similarity with this prototype CCHFV strain. Altogether, the mAbs exhibited eight different reactivity patterns, including some mAbs that recognized only M2 virus strains and others that recognized all strains tested. Of the seven mAbs known to neutralize IbAr10200 potently *in vitro* (Bertolotti-Ciarlet *et al.*, 2005), only 11E7 bound to all six virus strains. Of the five mAbs described previously to be

able to protect at least 70 % of suckling mice challenged with IbAr10200 (Bertolotti-Ciarlet *et al.*, 2005), 11E7 and 8F10 could bind to all six virus strains. It is important to note that, in each experiment, we used the parental IbAr10220 strain as a positive control (as it was recognized by all of the mAbs) and mock-transfected cells as a negative control (see Supplementary Fig. S3, available in JGV Online). These results suggest that there are significant antigenic differences between CCHFV strains that may not correlate well with genotypic or geographical characteristics. In addition, a number of epitopes to which neutralizing or protective mAbs can be directed are not highly conserved. However, at

Constructs	11E7 recognition
	+
971 1644	+
971 1224	-
1230 1383	-
1380 1644	+
1380 1472	-
1465 1563	-
1545 1642	-
1380 1528	-
1505 1644	-
1380 1566	+
1443 1566	+

Fig. 3. Mapping of the epitope recognized by neutralizing mAb 11E7. A schematic representation of the different IbAr10200 G_C fragments utilized to map the 11E7 epitope is shown. All of the constructs were expressed in mammalian cells and included a V5 epitope tag at the C terminus to control for expression. The numbers at the end of each construct represent the amino acid numbers based on the full-length IbAr10200 M segment.

least one broadly cross-reactive, potentially neutralizing mAb that can protect mice from a lethal CCHFV challenge (11E7) was identified.

Mapping of the 11E7 mAb epitope

As the neutralizing mAb 11E7 was able to recognize G_C by Western blot under non-reducing conditions, we were able to partially map its epitope by testing its ability to recognize fragments of G_C produced in HEK-293T cells. This is of relevance because mAb 11E7 protects mice *in vivo* from challenge with CCHFV strain IbAr10200 (Bertolotti-Ciarlet *et al.*, 2005). Passive immunization can be effective for the treatment of CCHFV infection in humans, emphasizing the importance of identification of neutralizing antibodies and the epitopes to which they bind (Vassilenko *et al.*, 1990).

We found that a G_C construct lacking the transmembrane and cytoplasmic domains was recognized by mAb 11E7 (Fig. 3). Therefore, we constructed three fragments that covered the length of the G_C ectodomain (C1, C2 and C3). All fragments contained a V5 epitope tag at the C terminus to allow detection of the fragment and to confirm their expression (Fig. 4). Most of the constructs, when expressed, formed SDS-resistant oligomers to some extent (Fig. 4). However, the relevance of this oligomerization is not clear, as the fragments represent only small portions of the protein and may therefore aggregate. Nonetheless, of these three fragments, only construct C3, located at the C terminus of the G_C ectodomain, was recognized by 11E7. Therefore, we focused our attention on this area, further dividing it into three new fragments (C3A, C3B and C3C). The antibody recognized none of these fragments. Next, we decided to divide the C3 fragment into two overlapping regions (C3.1 and C3.2); however, this resulted in disruption of the 11E7 epitope (Figs 3 and 4). Therefore, we performed a small deletion within the C3 C terminus (C3-T1). The antibody recognized this construct. Additionally, a small deletion of the N terminus of the C3 region also yielded a fragment recognized by mAb 11E7 (C3-T2) (Figs 3 and 4). Therefore,

we conclude that the neutralizing epitope of mAb 11E7 is contained between aa 1443 and 1566 of the M segment of IbAr10200 strain, a highly conserved region of the protein (Figs 3 and 4).

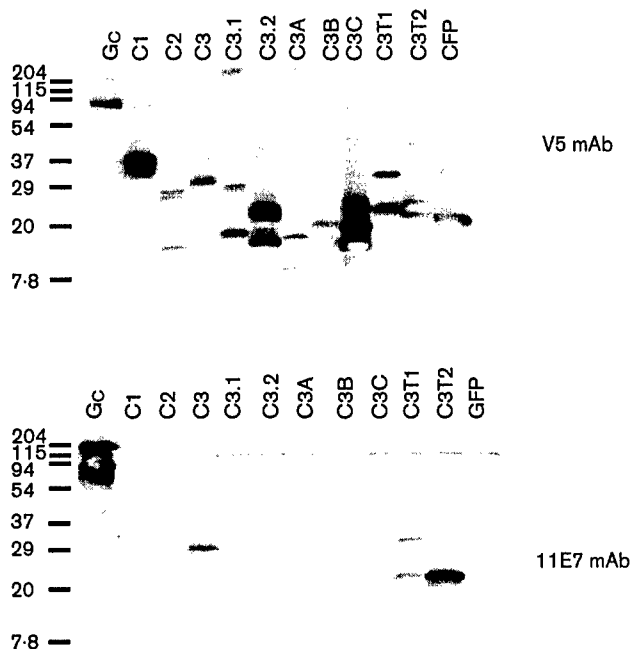


Fig. 4. Western blot analyses for mapping of mAb 11E7 epitope. Western blotting was performed by using lysates of HEK-293T cells transfected with some of the constructs shown in Fig. 3 and developed by using mAb 11E7 in parallel with a mAb for the V5 tag (Invitrogen). Some of the smaller fragments ran as both monomers and oligomers in SDS-PAGE. Molecular markers are shown in kDa (Prestained SDS-PAGE standards, broad range; Bio-Rad). GFP, Green fluorescent protein.

Conclusion

In summary, we report the first description of CCHFV glycoprotein antigenic structure and relatedness, as well as initial mapping of a cross-reactive neutralizing epitope present on divergent CCHFV strains. CCHFV strains can exhibit considerable genetic variability, with the mucin-like domain in G_N in particular being highly divergent. We also found a considerable amount of antigenic variability, which may not follow phylogenetic groupings of CCHFV strains. Even the highly conserved G_C protein exhibited antigenic variability, suggesting that CCHFV glycoproteins are subject to immune selection. Nonetheless, at least some epitopes to which neutralizing and/or protective antibodies bind are conserved between divergent CCHFV strains, and definition of these antibody-binding sites may be useful for vaccine design.

ACKNOWLEDGEMENTS

We thank Aura Garrison and Donald Pijak for expert technical assistance. This work was supported in part by training grant NIH T32 AI055400, Department of Defense Peer Reviewed Medical Research Program grant PRMRP PR033269 and R21-AI-063308.

REFERENCES

- Alexander, W. A., Moss, B. & Fuerst, T. R. (1992). Regulated expression of foreign genes in vaccinia virus under the control of bacteriophage T7 RNA polymerase and the *Escherichia coli* lac repressor. *J Virol* 66, 2934–2942.
- Andersson, A. M. & Pettersson, R. F. (1998). Targeting of a short peptide derived from the cytoplasmic tail of the G1 membrane glycoprotein of Uukuniemi virus (*Bunyaviridae*) to the Golgi complex. *J Virol* 72, 9585–9596.
- Andersson, A. M., Melin, L., Bean, A. & Pettersson, R. F. (1997a). A retention signal necessary and sufficient for Golgi localization maps to the cytoplasmic tail of a *Bunyaviridae* (Uukuniemi virus) membrane glycoprotein. *J Virol* 71, 4717–4727.
- Andersson, A. M., Melin, L., Persson, R., Raschperger, E., Wikström, L. & Pettersson, R. F. (1997b). Processing and membrane topology of the spike proteins G1 and G2 of Uukuniemi virus. *J Virol* 71, 218–225.
- Bertolotti-Ciarlet, A., Smith, J., Strecker, K. & 7 other authors (2005). Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. *J Virol* 79, 6152–6161.
- Blackburn, N. K., Besselaar, T. G., Shepherd, A. J. & Swanepoel, R. (1987). Preparation and use of monoclonal antibodies for identifying Crimean-Congo hemorrhagic fever virus. *Am J Trop Med Hyg* 37, 392–397.
- Chen, S.-Y. & Compans, R. W. (1991). Oligomerization, transport, and Golgi retention of Punta Toro virus glycoproteins. *J Virol* 65, 5902–5909.
- Chen, S.-Y., Matsuoka, Y. & Compans, R. W. (1991). Golgi complex localization of the Punta Toro virus G2 protein requires its association with the G1 protein. *Virology* 183, 351–365.
- Chinikar, S., Persson, S.-M., Johansson, M. & 7 other authors (2004). Genetic analysis of Crimean-Congo hemorrhagic fever virus in Iran. *J Med Virol* 73, 404–411.
- Chumakov, M. P., Butenko, A. M., Shalunova, N. V. & 10 other authors (1968). New data on the viral agent of Crimean hemorrhagic fever. *Vopr Virusol* 13, 377 (in Russian).
- Chumakov, M. P., Smirnova, S. E. & Tkachenko, E. A. (1970). Relationship between strains of Crimean haemorrhagic fever and Congo viruses. *Acta Virol* 14, 82–85.
- Felsenstein, J. (1997). An alternating least squares approach to inferring phylogenies from pairwise distances. *Syst Biol* 46, 101–111.
- Gerrard, S. R. & Nichol, S. T. (2002). Characterization of the Golgi retention motif of Rift Valley fever virus G_N glycoprotein. *J Virol* 76, 12200–12210.
- Hewson, R., Chamberlain, J., Mioulet, V. & 9 other authors (2004a). Crimean-Congo haemorrhagic fever virus: sequence analysis of the small RNA segments from a collection of viruses world wide. *Virus Res* 102, 185–189.
- Hewson, R., Gmyl, A., Gmyl, L., Smirnova, S. E., Karganova, G., Jamil, B., Hasan, R., Chamberlain, J. & Clegg, C. (2004b). Evidence of segment reassortment in Crimean-Congo haemorrhagic fever virus. *J Gen Virol* 85, 3059–3070.
- Hoogstraal, H. (1979). The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. *J Med Entomol* 15, 307–417.
- Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G. & Gibson, T. J. (1998). Multiple sequence alignment with Clustal X. *Trends Biochem Sci* 23, 403–405.
- Morais, V. A., Crystal, A. S., Pijak, D. S., Carlin, D., Costa, J., Lee, V. M.-Y. & Doms, R. W. (2003). The transmembrane domain region of nicastrin mediates direct interactions with APH-1 and the γ -secretase complex. *J Biol Chem* 278, 43284–43291.
- Morikawa, S., Qing, T., Xinqin, Z., Saijo, M. & Kurane, I. (2002). Genetic diversity of the M RNA segment among Crimean-Congo hemorrhagic fever virus isolates in China. *Virology* 296, 159–164.
- Niwa, H., Yamamura, K. & Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108, 193–199.
- Olaleye, O. D., Tomori, O. & Schmitz, H. (1996). Rift Valley fever in Nigeria: infections in domestic animals. *Rev Sci Tech* 15, 937–946.
- Onishchenko, G. G., Lomov, Iu. M., Markov, V. I. & 12 other authors (2000). The laboratory diagnosis of an outbreak of hemorrhagic fever at Oblivskaya village, Rostov Province: proof of the etiological role of the Crimean-Congo hemorrhagic fever virus. *Zh Mikrobiol Epidemiol Immunobiol* 32–36 (in Russian).
- Onishchenko, G. G., Efremenko, V. I., Kovalev, N. G. & 12 other authors (2001a). Specific epidemiologic features of Crimean haemorrhagic fever in Stavropol' region in 1999–2000. *Zh Mikrobiol Epidemiol Immunobiol* 86–89 (in Russian).
- Onishchenko, G. G., Markov, V. I., Merkulov, V. A., Vasil'ev, N. T., Berezhnoi, A. M., Androshchuk, I. A. & Maksimov, V. A. (2001b). Isolation and identification of Crimean-Congo hemorrhagic fever virus in the Stavropol territory. *Zh Mikrobiol Epidemiol Immunobiol* 7–11 (in Russian).
- Page, R. D. M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12, 357–358.
- Papa, A., Ma, B.-J., Kouidou, S., Tang, Q., Hang, C.-S. & Antoniadis, A. (2002). Genetic characterization of the M RNA segment of Crimean Congo hemorrhagic fever virus strains, China. *Emerg Infect Dis* 8, 50–53.
- Sanchez, A. J., Vincent, M. J. & Nichol, S. T. (2002). Characterization of the glycoproteins of Crimean-Congo hemorrhagic fever virus. *J Virol* 76, 7263–7275.

A. A. Ahmed and others

- Schmaljohn, C. S. (1996). *Bunyaviridae*: the viruses and their replication. In *Fields Virology*, 3rd edn, pp. 1447–1471. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott-Raven.
- Shepherd, A. J., Swanepoel, R., Shepherd, S. P., Leman, P. A., Blackburn, N. K. & Hallett, A. F. (1985). A nosocomial outbreak of Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part V. Virological and serological observations. *S Afr Med J* 68, 733–736.
- Simmons, G., Wool-Lewis, R. J., Baribaud, F., Netter, R. C. & Bates, P. (2002). Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence. *J Virol* 76, 2518–2528.
- Simpson, D. I., Knight, E. M., Courtois, G., Williams, M. C., Weinbren, M. P. & Kibukamusoke, J. W. (1967). Congo virus: a hitherto undescribed virus occurring in Africa. I. Human isolations – clinical notes. *East Afr Med J* 44, 86–92.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25, 4876–4882.
- Tignor, G. H., Smith, A. L., Casals, J., Ezeokoli, C. D. & Okoli, J. (1980). Close relationship of Crimean hemorrhagic fever-Congo (CHF-C) virus strains by neutralizing antibody assays. *Am J Trop Med Hyg* 29, 676–685.
- Tippmann, H.-F. (2004). Analysis for free: comparing programs for sequence analysis. *Brief Bioinform* 5, 82–87.
- Vassilenko, S. M., Vassilev, T. L., Bozadjiev, L. G., Bineva, I. L. & Kazarov, G. Z. (1990). Specific intravenous immunoglobulin for Crimean-Congo haemorrhagic fever. *Lancet* 335, 791–792.
- Vincent, M. J., Sanchez, A. J., Erickson, B. R., Basak, A., Chretien, M., Seidah, N. G. & Nichol, S. T. (2003). Crimean-Congo hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1. *J Virol* 77, 8640–8649.
- Whitehouse, C. A. (2004). Crimean-Congo hemorrhagic fever. *Antiviral Res* 64, 145–160.
- Williams, R. J., Al-Busaidy, S., Mehta, F. R. & 7 other authors (2000). Crimean-Congo haemorrhagic fever: a seroepidemiological and tick survey in the Sultanate of Oman. *Trop Med Int Health* 5, 99–106.
- Wood, O. L., Lee, V. H., Ash, J. S. & Casals, J. (1978). Crimean-Congo hemorrhagic fever, Thogoto, Dugbe, and Jos viruses isolated from ixodid ticks in Ethiopia. *Am J Trop Med Hyg* 27, 600–604.
- Woodall, J. P., Williams, M. C. & Simpson, D. I. (1967). Congo virus: a hitherto undescribed virus occurring in Africa. II. Identification studies. *East Afr Med J* 44, 93–98.